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BACTERIAL ZOOGLOEA FORMATION

Richard F. Unz, et al

Pennsylvania State University

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16. ABSTRACT

Activated sludge flocs suspended in wet mounts on microscope slides were observed to sprout, finger-like, bacterial zoogloeae as a consequence of the outgrowth of bacteria from flocs. The rate of extension of finger-like zoogloeae was typically 5.1 to 15.0 μm per hr and mean cell doubling time was estimated to be approximately 2 hrs. Photomicrographic and fluorescent antibody studies revealed that the bacterial zoogloeae consisted of the progeny of specific zoogloea-forming bacteria. Purified exopolymers of Zoogloea strains and domestic activated sludge contained two amino sugars, one of which was identified as glucosamine. Zoogloea exopolymer was not fibrilar or cellulosic and contained approximately 17 to 19 per cent amino sugar and about one per cent hexoses, uronic acids and ether soluble substances on a dry weight of polymer basis. Amino sugar production was found to parallel zoogloea formation by Zoogloea sp. Calcium ion appeared to augment flocculation of bacterial cells capable of undergoing natural coalescence. Two cell types, described as rough and smooth colony-forming, were found in some strains of Zoogloea. Rough cells readily flocculated in agitated cultures whereas smooth cells produced relatively turbid cultures under similar growth conditions. A predominance of one of the two types could influence the degree of flocculation by Zoogloea cultures.

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BACTERIAL ZOOGLOEA FORMATION

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FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, noise and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment -- air, water, and land. The National Environmental Research Centers provide this multidisciplinary focus through programs engaged in

- studies on the effects of environmental contaminants on man and the biosphere, and
- a search for ways to prevent contamination and to recycle valuable resources.

In an effort to achieve the foregoing objectives, this project has attempted to identify the factors involved in bacterial zoogloea formation. A thorough understanding of the process of floc formation will be an invaluable aid to future design engineering and wastewater treatment research.

A. W. Breidenbach, Ph.D.
Director
National Environmental
Research Center, Cincinnati

ABSTRACT

Activated sludge flocs prepared in wet mounts on microscope slides were observed to sprout typical, finger-like, bacterial zoogloeae by a process of outgrowth. The rate of extension of finger-like zoogloeae was typically 5.1 to 15.0 μm per hr and mean cell doubling time was estimated to be approximately 2 hrs. Finger-like zoogloea formation appeared to be an aerotactic or chemotactic phenomenon. Photomicrographic and fluorescent antibody studies showed that the bacterial zoogloeae consisted essentially of the progeny of specific zoogloea-forming bacteria.

Purified exopolymers obtained from axenic cultures of Zoogloea strains and domestic activated sludge contained two amino sugars, one of which was identified as glucosamine. Zoogloea exopolymer consisted of approximately 17 to 19 per cent amino sugar on a dry weight basis. Hexoses, uronic acids and ether soluble substances were only about one per cent of the dry weight of polymer and the polymer was not fibrilar or affected by reaction with cellulase. Amino sugar production was found to parallel zoogloea formation by Zoogloea sp.

Calcium ion appeared to augment flocculation of bacterial cells which were capable of undergoing natural coalescence. Two types of cells, described as rough and smooth colony-forming, were found in some strains of Zoogloea. Rough cells readily flocculated in agitated cultures whereas smooth cells produced relatively turbid cultures under similar growth conditions. A predominance of one of the two types could influence the degree of flocculation by Zoogloea cultures.

This report was submitted in fulfillment of Project Number 17050 DBI by the Pennsylvania State University under the sponsorship of the Environmental Protection Agency. Work was completed as of October 1973.

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The absence of microfibrils in the exopolymer of *Zoogloea* strains was determined from electron photomicrographs taken by Mr. Thomas Rucinsky, Department of Microbiology, The Pennsylvania State University.

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Section I

CONCLUSIONS

1. Bacterial zoogloeae of the finger-like type are formed during growth and multiplication of zoogloea-forming bacteria which are present in activated sludge flocs.

2. The formation of the finger-like zoogloeae seems influenced by aerotaxis, chemotaxis or a combination of these phenomena.

3. The gelatinous matrix of a zoogloea does not inhibit movement of component bacterial cells and it is even possible for cells to escape from the zoogloeal structures.

4. The gelatinous matrix is an exopolymer containing two amino sugars, one of which has been identified as glusosamine.

5. Amino sugars are present in fairly constant and similar amounts in exopolymer of axenic Zoogloea strains. Amino sugars may be used as an indirect measure of exopolymer and these sugars are from 18-19 per cent of the dry weight of purified polymer.

6. Two amino sugars were the major reducing substances found in the purified exopolymer of domestic activated sludge and one of these was identified as glucosamine.

7. Amino sugars appear in association with the flocculation and zoogloea formation by Zoogloea strains. Flocculation of cells begins in the late logarithmic phase of the growth cycle and amino sugars are produced well into the stationary phase.

8. Natural finger-like zoogloeae consist essentially of the progeny of a single strain of zoogloea-forming bacterium which most probably belongs to the genus Zoogloea.

9. Calcium ion may, under appropriate conditions, augment flocculation of cells of Zoogloea sp. It does not appear that cells which do not naturally flocculate are positively affected by calcium ion.

10. Smooth and rough colony-forming cells exist in cultures of Zoogloea strains and it is the rough type cells which strongly flocculate.

11. A depressed oxidation-reduction potential does not appear to stimulate formation of zoogloeae by axenic Zoogloea strains. In mixed culture, lowered oxidation-reduction potential creates an environment conducive to anaerobic decomposition of organic matter and the metabolic by products (volatile acids) may be utilized by zoogloea-forming bacteria under microaerophilic and aerobic conditions.

Section II

RECOMMENDATIONS

The research described in this report was limited essentially to experimentation with axenic Zoogloea strains and aerobic wastewater sludges. The study focused on the bacteria which form finger-like zoogloeae in liquid culture medium. Although it is apparent that these organisms are present in activated sludge and may be active under appropriate conditions, it is not known if they contribute significantly to the formation of activated sludge. A means to quantify viable Zoogloea spp. in sludges is desparately needed in order to determine their numbers in flocs originating under different cultural conditions. A screening of numerous bacteria, freshly isolated from activated sludge, should be undertaken to identify the zoogloeal organisms and perform chemical analyses on the exopolymers. Since it may be expected that exopolymers of sludges are the products of resident microorganisms, it may be enlightening to "finger print," by chemical characterization, the polymers of various sludges. Such an undertaking may lead to the development of a new procedure for determining the quality of sludges in relation to wastewater treatment performance and be of assistance in maintaining control of aerobic biological treatment systems. Samples of mixed liquor suspended solids should be obtained from several different biological wastewater treatment plants and examined for the presence of finger-like zoogloeae in flocs. In this way, it may be possible to establish a correlation between finger-like zoogloeae in activated sludge and some feature(s) or condition(s) of treatment plant operations. The finger-like zoogloeae may prove to be a sensitive indicator of changing environmental conditions in the activated sludge process.

The important mechanisms of flocculation and deflocculation in activated sludge have not been resolved and further study in this area is highly desirable. The discovery of rough and smooth colony-forming cells in cultures of Zoogloea strains has far reaching implications because if these bacteria are important structural determinants of activated sludge new approaches to the study of wastewater bioflocculation will be in order. It would be of interest to learn if other bacteria isolated from activated sludge give rise to rough and smooth cells.

Finally, refined growth experiments need to be performed with Zoogloea strains. Chemostat studies employing a suitable substrate would be valuable for determining minimum nutrient levels required to sustain maximum growth of the Zoogloea sp. Continuous culture should be used to find the conditions under which Zoogloea sp. will grow and flocculate in chemically defined culture media and wastewaters.

Section III

INTRODUCTION

Bacterial zoogloeae consist of bacterial cells embedded in a confining gelatinous matrix. The importance of the zoogloeal masses to the structure of activated sludge has been emphasized in the past (10, 30). However, other mechanisms have been advanced to explain bioflocculation leading to the formation of activated sludge (13, 15, 43, 44, 53, 57). The validity of certain flocculation theories has been challenged (16, 57, 63). It should be noted that zoogloea formation and bacterial flocculation are not always similar (23, 64).

The floc-forming pseudomonad, Zoogloea ramigera, has been intimated as an important functional bacterium in the activated sludge and trickling filter wastewater treatment process (10, 11, 12, 29, 33). Z. ramigera was originally described and named in 1867 by Itzigsohn (31). The species designated was made on the basis of the finger-like or tree-like zoogloeae which distinguished the organism in mixed cultures from another less obvious zoogloea-forming bacterium, Z. termo. Concern about the taxonomic validity of Z. ramigera and its significance in aerobic biological wastewater treatment has inspired several laboratory studies on microorganisms identified as Z. ramigera. The information obtained from these investigations has been largely inconclusive in clarifying the position of Z. ramigera with respect to the important aforementioned issues. Conflicting reports on the characteristics of assumed pure cultures of Z. ramigera increased confusion about the organism. It remained to obtain bacterial cells from wastewater zoogloeae in a manner which would permit definite statements about the origin of isolates. Such experiments were performed by Unz and Dondero (65) who showed that the majority of the bacterial cells present in finger-like bacterial zoogloeae were able to form in axenic cultures zoogloeae which were similar in appearance to the natural wastewater forms. The axenic cultures of zoogloea-forming bacteria were characterized and found to be dentrifying, ureolytic pseudomonads which could hydrolyze gelatin and grow readily on short chain fatty acids but were inactive on carbohydrates (65, 66). These bacteria were identified as strains of Zoogloea which, in many ways, were dissimilar to the non-zoogloeal, floc-forming Z. ramigera I-16-M of Crabtree and McCoy (14) and the Z. ramigera 115 of Freidman and Dugan (23). Unz (64) suggested adoption of Z. ramigera 106 (ATCC 19544) as the neotype of Z. ramigera to replace Z. ramigera I-16-M (ATCC 19623).

Unz and Dondero (67) have isolated nonzoogloea-forming bacteria from finger-like and amorphous wastewater zoogloeae. They found several of these bacteria to be active in degradation of carbohydrates and they proposed that some wastewater zoogloeae may consist of certain bacteria responsible for the formation of zoogloeal matrix as well as non-zoogloeal bacteria which become entrapped in the zoogloeal matter during

expansion of the floc. No definite answers are available to support these hypotheses and further research on the mechanisms of bacterial zoogloea formation is needed. In an attempt to learn more about the nature of bacterial zoogloea formation, a research program was undertaken to study the development of bacterial zoogloeae in mixed cultures and elucidate the factors stimulatory to growth and zoogloea production by Zoogloea sp.

Section IV

MATERIALS AND METHODS

CINEMATOGRAPHY

Preparation of specimens for photographing bacterial zoogloea formation. A suspension of activated sludge flocs was mounted on a glass microscope slide and covered with a 22 x 44 mm coverslip which was sealed with vaseline. Approximately one-third to one-half of the total volume beneath the coverslip was occupied by intentionally entrapped air. A satisfactory density of sludge particles for microscopic observation was achieved by trial and error. It was found that very high and very low concentrations of sludge particles were unsuitable for obtaining good bacterial zoogloea formation. Slides were observed at ambient temperature (28 C $\pm$ 1 C).

Cinematographic techniques.
Wet mounts of activated sludge flocs were examined using a Zeiss Universal microscope equipped with phase optics (Carl Zeiss, Inc., New York; Fig. 1, A). Time lapse cinematography was performed using an Arriflex model S 16 mm motion picture camera with an Arriflex DOM single frame drive motor (Arriflex Corp., West Germany; Fig. 1, B). The camera was attached to a Wild microscope stand (Wild Microscope Co., Heerbrugg, Switzerland; Fig. 1, C). Continuous illumination of slides was avoided by the use of an Ilex electronic shutter no. 4 mounted over the field diaphragm (Ilex Optical Co., Rochester, N. Y.; Fig. 1, D). Exposures were taken at the rate of four to six frames per minute. Timing was controlled by a time lapse intervalometer (Camera Equipment Co., Inc., New York; Fig. 1, E). Coordination of the camera and the electronic shutter was implemented by a camera-shutter function timer fabricated at the Pennsylvania State University. Constant voltage for the light source was maintained with a Solatron model 2KVA voltage regulator (Sola Electric Co., Elk Grove Village, Ill.)

Still photographs were taken with a 35 mm Zeiss Ikon attachment camera.

BACTERIA AND BACTERIAL CELL ENUMERATION

Axenic cultures of the following bacteria were employed in experiments: (a) Zoogloea strains 9, 21 (ATCC 19122), 106 (ATCC 19544), 201 (ATCC 19325), 216 (ATCC 19123), 235 (ATCC 19324), 239 (ATCC 19173), I-16-M (ATCC 19623), Z. ramigera 115, which was kindly supplied by P. R. Dugan, The Ohio State University, Columbus, Ohio, freshly isolated Zoogloea strains (68) and; (b) various bacteria from the stock culture collection of the Department of Microbiology, The Pennsylvania State University.

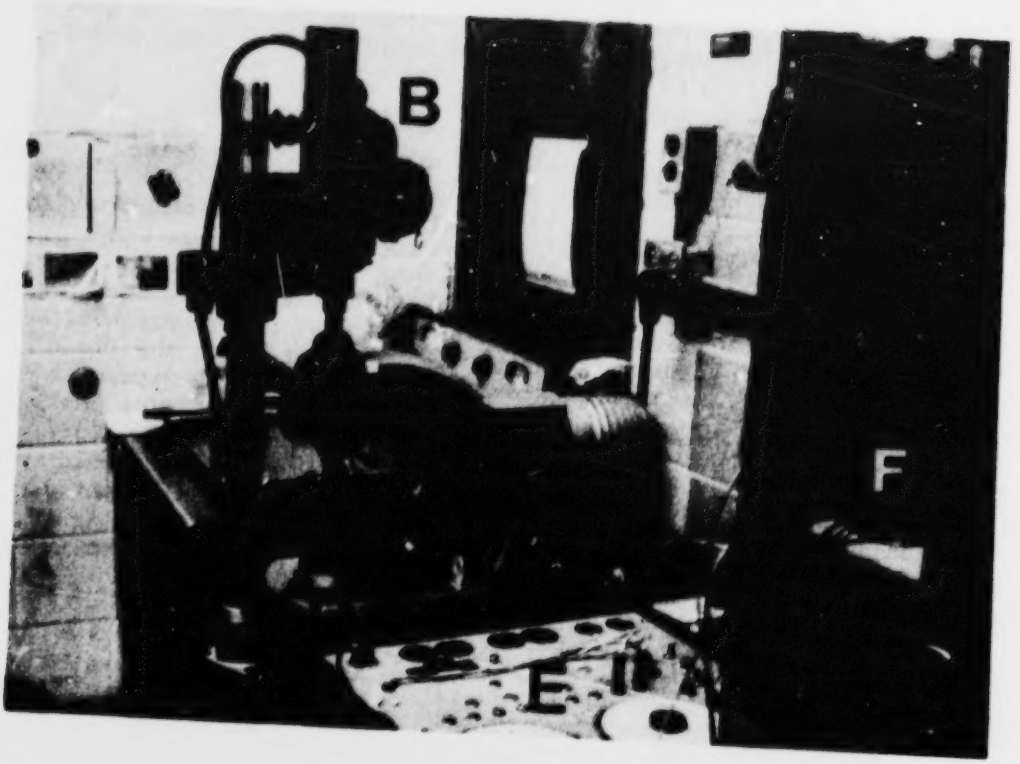

Fig. 1. Equipment Used for Time-Lapse Cinematography. A, Zeiss Universal microscope; B, Arriflex DOM 16 mm camera; C, Wild microscope stand; D, Flex electronic shutter; E, time-lapse intervalometer; F, camera shutter function timer.

Stock cultures of Zoogloea strains were maintained in liquid Casitone-yeast autolysate (CY) medium containing per liter of distilled water: Casitone (Difco), 5.0 g. and yeast autolysate (Charles Pfizer and Co., Inc., New York), 1.0 g.

Direct cell counts were made at 500 X using a Petroff-Hausser counting chamber with the aid of a Zeiss Universal microscope.

Viable cell counts were determined by spread plating appropriate dilutions of culture fluids on CY medium. Plates were incubated at 23 C for 72 hr.

Dry weight of twice washed biomass was estimated by drying samples at 105 C and cooling to constant weight.

Cell nitrogen was determined by the semimicro-Kjeldahl method of McKenzie and Wallace (42).

Optical density measurements were performed at 500 nm using a Bausch & Lomb Spectronic 70 spectrophotometer (Bausch & Lomb Co., Rochester, N. Y.).

SEROLOGY

Preparation of inoculum and serum production.

Z. ramigera 106 was cultured in a medium containing per liter of distilled water: $(NH_4)_2SO_4$, 0.264 g; K_2HPO_4, 0.087 g; $MgSO_4$, 0.120 g; $CaSO_4$, 0.136 g; sodium lactate, 1.0 g; Casitone, 0.10 g; yeast autolysate, 0.02 g. Incubation was at 20 C on a gyrotary shaking machine for 48 hr. Four liters of culture fluid were centrifuged at 5000 X g for 10 min to recover cell mass. Cells were washed twice in distilled water, suspended in a 50 ml volume of distilled water and the suspension was adjusted to pH 10.0 using 1 N NaOH. The suspension was boiled for 3 min, cooled, pH readjusted to 10.0 and boiled for another 3 min. Finally, cells were centrifuged at 27,000 X g for 10 min and washed twice in distilled water. Microscopic examination of treated cells in wet mounts containing India ink revealed clean preparations of cells devoid of exocellular gelatinous substances. A quantity of the clean cells equal to 1.0 mg Kjeldahl nitrogen per ml was mixed with an equal volume of mineral oil. The mixture served as the inoculum for the production of antiserum. Domestic rabbits received 1 ml subcutaneous injections in each flank. After 1 month, rabbits were given intravenous injections consisting of 1 ml of the cell preparation equal to 0.2 mg Kjeldahl nitrogen per ml. One week following intravenous injections, antiserum was obtained by bleeding the animals from the heart.

Preparation and use of fluorescein labeled antibody.
Serum globulins were fractionated from the rabbit antiserum and buffered according to the procedure of Romano and Geason (55). Buffered globulins were mixed with 0.015 mg of fluorescein isothiocyanate per mg of protein as suggested by Olson (51). The mixture was allowed to react overnight at 5 C under continuous mechanical stirring followed by dialysis against buffered saline consisting of 0.15 M NaCl and 0.1 M K_2HPO_4; final pH 7.2. Dialysis was continued until no fluorescein was observed in the dialysate. The labeled globulin was sterilized by passage through a 0.45 µm membrane filter and frozen. Labeled globulin was used in studies of cross reactions with various known strains of Zoogloea and other axenic cultures of bacteria as well as unknown bacteria isolated from activated sludge. The bacteria were isolated on CY agar medium and activated sludge agar prepared according to the method of Prakasam and Dondero (54). Bacteria were spread on microscope slides, air dried, fixed in ethanol, and permitted to react with antiserum for 15 min at 28 C. Following incubation, slides were washed with buffered saline, covered with mounting fluid (mixture of equal volumes of glycerol and buffered saline) and coverslips, and sealed with nail polish. Slides were examined by dark field microscopy using a Zeiss Universal microscope equipped with an Osram HBO 200 watt ultraviolet light source and exciter filter UG 5 and barrier filter 47/65. A cross reaction with Z. ramigera 115, a bacterium of questionable identity in our opinion, necessitated further refinement of the Z. ramigera 106 antiserum before the antiserum could be used in the analysis of unknown bacterial cells. Cells of Z. ramigera 115 were cultivated in CY medium, harvested and washed as previously described, and suspended in saline to give a final concentration of 1 mg of cells per ml. To insure the specificity of the antiserum, equal volumes of Z. ramigera 115 in saline and labeled antiserum were incubated at 37 C for one hour. Cells with sorbed antibody were removed from the antiserum by centrifugation at 27,000 X g for 10 min. followed by filtration of the purified antiserum through a 0.45 µm membrane filter.

Highly specific Z. ramigera 106 antiserum was used in diagnostic tests on the microbial film which formed at the surface of settled activated sludge stored in beakers at 28 C for 48 hr.

ZOOGLOEAL MATRIX (EXOPOLYMER)

Harvesting, purification, and hydrolysis.
Mass production of the zoogloeal matrix required for chemical analysis of the polymer was accomplished by batch culturing zoogloea-forming bacteria in 1 liter quantities of liquid medium on a reciprocating shaking machine at 20 C. The bacteria were cultivated on several kinds of media including CY, trypticase soy (BBL) and a basal medium (BM) which contained per liter of distilled water: $(NH_4)_2SO_4$, 0.264 g; K_2HPO_4, 0.087 g; $MgSO_4$, 0.006 g; and sodium lactate, 1.000 g to which was added $CaSO_4$, 0.136 g.

Zoogloeal flocs were harvested from 48-hr old cultures by centrifugation and washed twice in distilled water and suspended in 10 ml of distilled water. Exopolymers were dissolved either by adjusting the washed zoogloeae to pH 10 with 1.0 N NaOH or 0.02 M K_2HPO_4 and heating for 10 min in a boiling water bath or by employing a Waring blender to homogenize for 1 min 50 ml quantities of the zoogloeae suspended in 0.02 M K_2HPO_4 or distilled water. Cell residue was removed from the dissolved polymer by centrifugation at 27,000 X g for 10 min. The clear supernatant was dialyzed against distilled water for 24 hr at 5 C and concentrated with polyethylene glycol when necessary. The polymeric substance was precipitated by adding 0.2 g of cetyltrimethylammonium bromide (CTAB) to 25 ml of solution and dried on standing at 5 C overnight. The residue was dissolved in 0.5 M NaCl and centrifuged at 27,000 X g for 10 min. The clear supernatant was dialyzed against several volumes of distilled water over a 24 hr period at 5 C.

Purified, dissolved zoogloeal matrix (approximately 0.5 mg per ml) and concentrated HCl were mixed to produce a 6 N HCl solution which was dispersed to screw capped test tubes. The matrix was hydrolyzed in a boiling water bath for 0.25, 0.75, 2.0, 6.0, and 12.0 hr. The HCl was removed following hydrolysis by drying samples under a stream of air at 28 C.

Natural activated sludge was blended in distilled water to obtain exopolymer which was concentrated and hydrolyzed by the procedures described above.

Chromatography

Ion exchange chromatography. Zoogloeal matrix hydrolysates were fractionated in 0.9 by 33.0 cm columns containing Dowex 50 (X8; H^+ form) resin (J. T. Baker Chemical Co., Phillipsburg, N.J.) according to the method of Gardell (27). One milliliter fractions were collected at the rate of 1 to 2 ml per hr using a Warner-Chilcott model 1205 fraction collector (Warner-Chilcott Laboratories, Richmond, Calif.)

Paper chromatography. One dimensional descending paper chromatography of hydrolysates was performed using Whatman no. 1 filter paper and one of the following solvent systems: butanol-acetic acid-distilled water (12:3:5); butanol-pyridine-distilled water (3:2:1.5); and isopropanol-distilled water (4:1). Chromatograms were developed for detection of carbohydrates using a silver nitrate reagent (61) and 3.0 per cent p-anisidine·HCl in butanol (47). Amino sugars were detected by spraying chromatograms with 0.3 per cent ninhydrin in acetone.

Chemical Analyses. Hexosamine was determined by the modified Elson-Morgan method as described by Kabat and Mayer (34) employing D-glucosamine·HCl as the standard. Total reducing sugar was analyzed by the procedure of Nelson (48) with either D-glucose or D-glucosamine·HCl as the standard. Uronic acids were determined by the method of Dische (19).

Hexoses were measured using the phenol sulfuric acid method of Dubois et al. (20). N-acetyl form of hexosamines was determined by the method of Aminoff, Morgan and Watkins as given by Kabat and Mayer (34).

Volatile acids were determined using column partition chromatography (3).

Oxidation-reduction potential measurements were made using an Orion model 801 digital pH/mv meter (Orion Research Inc., Cambridge, Mass.) equipped with a platinum thimble electrode (Beckman Instrument Co., Fullerton, Calif.). ZoBell solution (28) was used to standardize the system for redox measurements.

Electron Microscopy. Specimens were examined with a Phillips model 300 electron microscope at 60-80 kv. Shadow and replica preparations were made as described below using a Balzers model BA 360M freeze etch device (Balzers, Principality of Leichtenstein).

Negative stain. One drop of sample was applied to a carbon-coated, 200 mesh grid and stained with either one or two per cent phosphotungstate at pH 7.0-7.2.

Shadowing. One drop of sample was placed on a carbon coated grid and either drained of excess water before air drying or permitted to dry completely without prior removal of fluid. Platinum carbon shadowing was done at an angle between 15 to 30 degrees.

Replica. Air dried samples on clean microscope glass slides were shadowed at between 15 to 70 degree angles, coated with carbon at 90 degree angle and floated from the slides. Cellular material was removed from replicas by treating with 70 percent H_2SO_4 and 0.5 per cent NaOCl for 30 to 60 min each with intermediate and final rinsing three times with distilled water.

BACTERIAL FLOCCULATION

Inorganic ions

Various salts were evaluated for their effect on the flocculation of bacteria. Sulfates and chlorides of Mg, Ca, Zn, Mn, Fe and Na as well as NaBr, and NaF were tested in concentrations of 0.001, 0.01, 0.1, and 1.0 mmol per liter. The culture medium used was the BM medium earlier described.

Carbon to nitrogen ratio

The influence of carbon to nitrogen ratios on bacterial flocculation was evaluated using BM medium supplemented with 1 mmol per liter of

$CaSO_4$. The sole carbon source, sodium lactate, and sole nitrogen source, ammonium sulfate, were varied in concentration to give a range of carbon to nitrogen ratios in the culture medium.

NATURAL BACTERIAL ZOOGLOEA FORMATION

Studies on enrichment cultures of branched and finger-like wastewater zoogloeae were conducted using beakers of activated sludge as previously described in this report and elsewhere (4, 63). Changes in the chemical properties of standing volumes of settled activated sludge were noted by measuring redox potential and the appearance of volatile acids. Mixed liquors used in these experiments were collected from wastewater treatment plants located at State College, Pa., and University Park, Pa. The effect of various reducing agents on the formation of bacterial zoogloeae were analyzed by first adding approximately 40 ml of warm molten agar containing a specific reducing compound to a 400-ml beaker and, following solidification of the agar at the bottom of the beaker, admitting 160 ml of mixed liquor over the agar layer. Specific reducing compounds employed in these experiments are shown in Table 17. The extent of bacterial zoogloea formation was determined by harvesting the zoogloea film which, if present, formed at the surface of beaker fluid in 48 to 72 hr. The film was carefully skimmed from the liquid surface with a glass microscope slide, transferred to a centrifuge tube, and concentrated by centrifugation at 10,000 X g for 10 min. Concentrated films were washed twice and suspended in distilled water. A measured volume of the suspension was retained for dry weight determination. The remaining mass was boiled in 0.02 M K_2HPO_4 at pH 10.0 as earlier described. The extracted gel was hydrolyzed for 45 min in 6 N HCl, air dried, and analyzed for amino sugar content.

Section V

RESULTS

CINEMATOGRAPHY

Finger-like and branched bacterial zoogloeae were found by microscopic observation to be present in slimes collected from trickling filters and in the films and scums which developed at the surface of mixed liquors stored in beakers (Fig. 2). The bacterial zoogloeae developed by outgrowth from cells present in activated sludge flocs which were contained in wet mounts on microscope slides. The growth of the zoogloeae were recorded by time-lapse cinematography and the film is available from the authors of this report. Salient excerpts of the motion picture are presented herein. Sequential development of finger-like zoogloeae from an activated sludge floc may be seen in Fig. 3. Apparently, individual bacterial cells are not rigidly fixed in the gelatinous matrix of the zoogloea and these may travel within the matrix creating "cell free" regions (Fig. 4). Bacterial cell 1 and bacterial cell 2 traveled within the zoogloea in opposite directions. Since cell 2 moved opposite to the direction of zoogloea extension and the rate of movement for both cell 1 (23 μm per hr) and cell 2 (29 μm perhr) was greater than the extension rate of the zoogloea (17 μm per hr), it does not appear that changes in positions of cells was due to physical displacement by stretching or intercalary expansion of the zoogloea. Bacteria were observed to move freely within zoogloeae on several other occasions and it was possible for cells to depart from a zoogloea (Fig. 5).

Apart from the effect of cell movement, cell vacancies in a zoogloea may result from death and lysis of bacteria. The finger-like zoogloea shown in Fig. 6 appears to contain bacteria only in the anterior region. The striking contrast between intact and lysed bacteria in the zoogloea is evident in Fig. 7.

Branched zoogloeae were frequently observed in natural slimes and in the scums which developed at the surface of mixed liquors stored in the laboratory. However, only rarely were branched zoogloeae seen to form in wet mounts of activated sludge flocs and the structures were never photographed in time lapse studies. However, formation of a bifurcate bacterial zoogloea (Z2) in a wet mount of activated sludge flocs was photographed with a still picture camera (Fig. 8). It can be seen that the lateral branch of zoogloea Z2 and zoogloea Z1 developed in a direction contrary to that of the main stem of zoogloea Z2 and zoogloea Z3. Possibly, zoogloea Z1 and the branch of zoogloea Z2 were influenced in their development by changes in the microenvironment of the microscope slide culture. Inspection of a composite view of several photographed microscope fields of a wet mount of activated sludge flocs (Fig. 9) revealed the tendency for bacterial zoogloeae to develop in a direction

Fig. 2. Natural, Finger-Like, Bacterial Zoogloeae. Specimen obtained from scum layer which developed on the surface of mixed liquor stored in a beaker; 48 hr, 28 C. Zoogloeae treated with India ink to accentuate matrix boundary. Phase contrast; wet mount. Bar equals 10 μm.

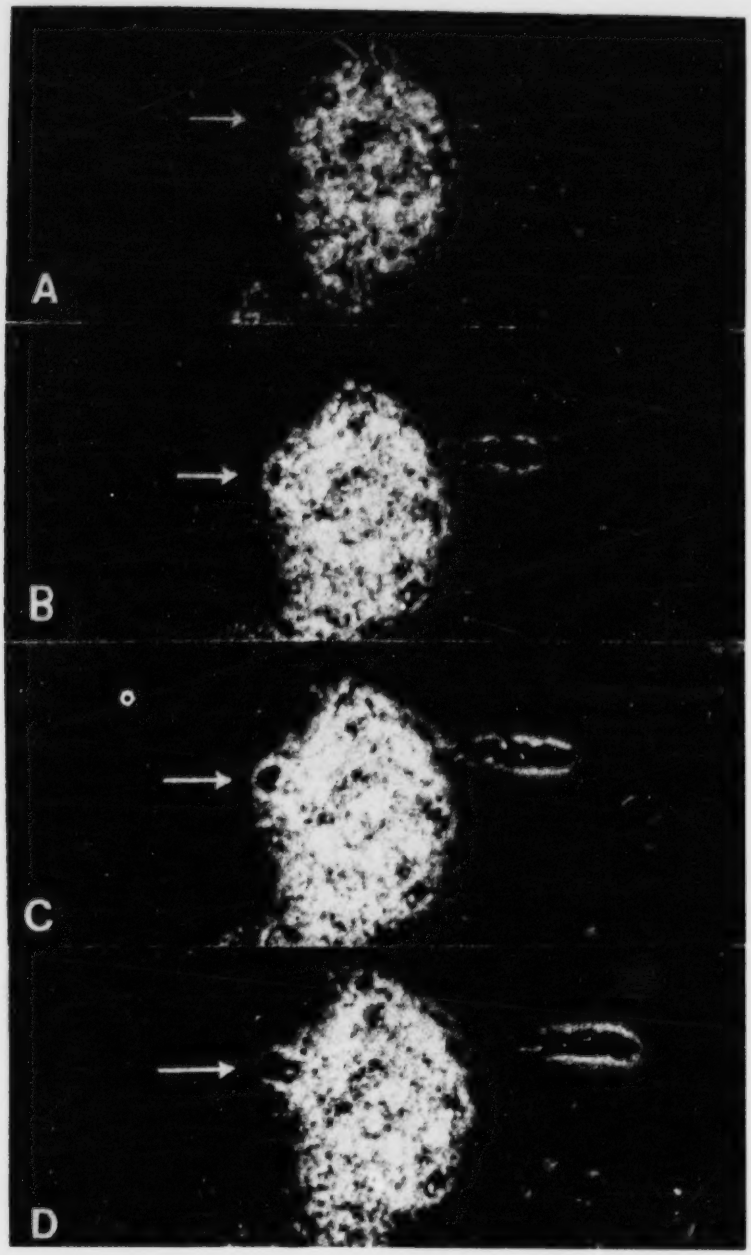

Fig. 3. Sequential Development of Two Bacterial Zoogloeae from an Activated Sludge Floc. Note appearance of one zoogloea seemingly from within floc (F, arrow). Time elapsed (min): A, 0; B, 64; C, 93; D, 138. Phase contrast; wet mount. Bar equals 10 μm.

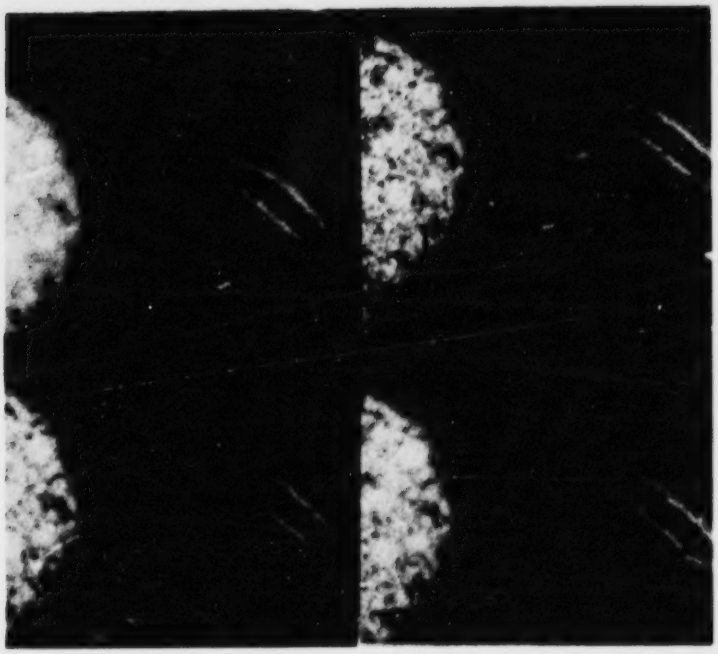

Fig. 4. Movement of Bacteria Within a Natural, Finger-Like, Bacterial Zoogloea. Time elapsed (min): A, 0; B, 5.25; C, 8.75; D, 21. Arrows indicate position of motile cells 1 and 2 within the zoogloea. Phase contrast; wet mount. Bar equals 10 µm.

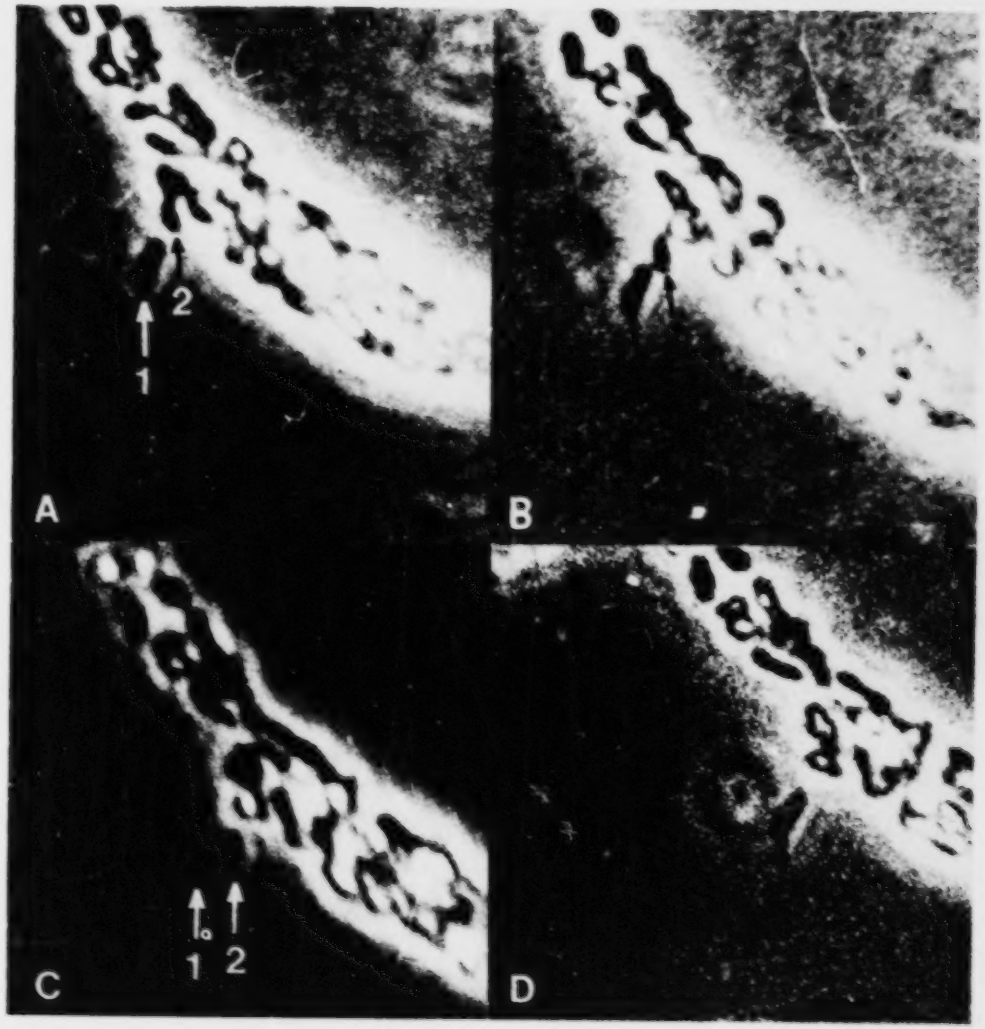

Fig. 5. Cell Departure from Natural, Finger-Like, Bacterial Zoogloea. Time elapsed (min): A, 0; B, 6.45; C, 7.45; D, 8.0. Arrows indicate position of cell 2 with respect to cell 1 until its escape from the zoogloea. Phase contrast; wet mount. Bar equals 5 μm.

Fig. 6. Natural, Finger-Like, Bacterial Zoogloea Exhibiting Vast, Cell-Free Regions. Cells (C) and background debris (D). Specimen obtained from scum layer which developed on the surface of mixed liquor stored in a beaker; 72 hr, 28 C. Zoogloea treated with India ink to accentuate matrix boundary. Phase contrast; wet mount. Bar equals 10 µm.

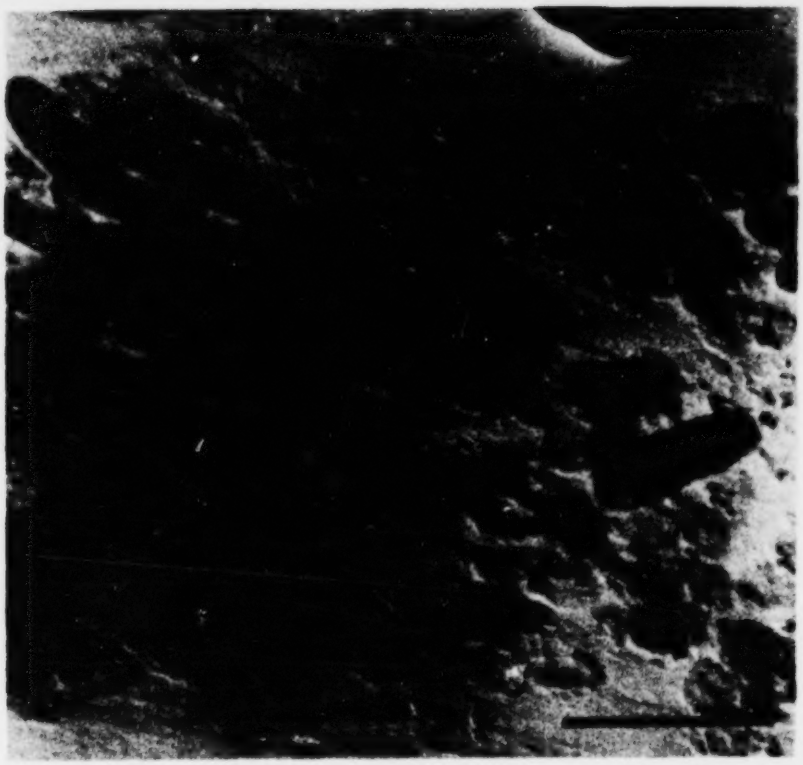

Fig. 7. Portion of Axenic, Finger-Like, Bacterial Zoogloea Showing Intact and Ghosted Cells. Electron photomicrograph. Bar equals 5 µm.

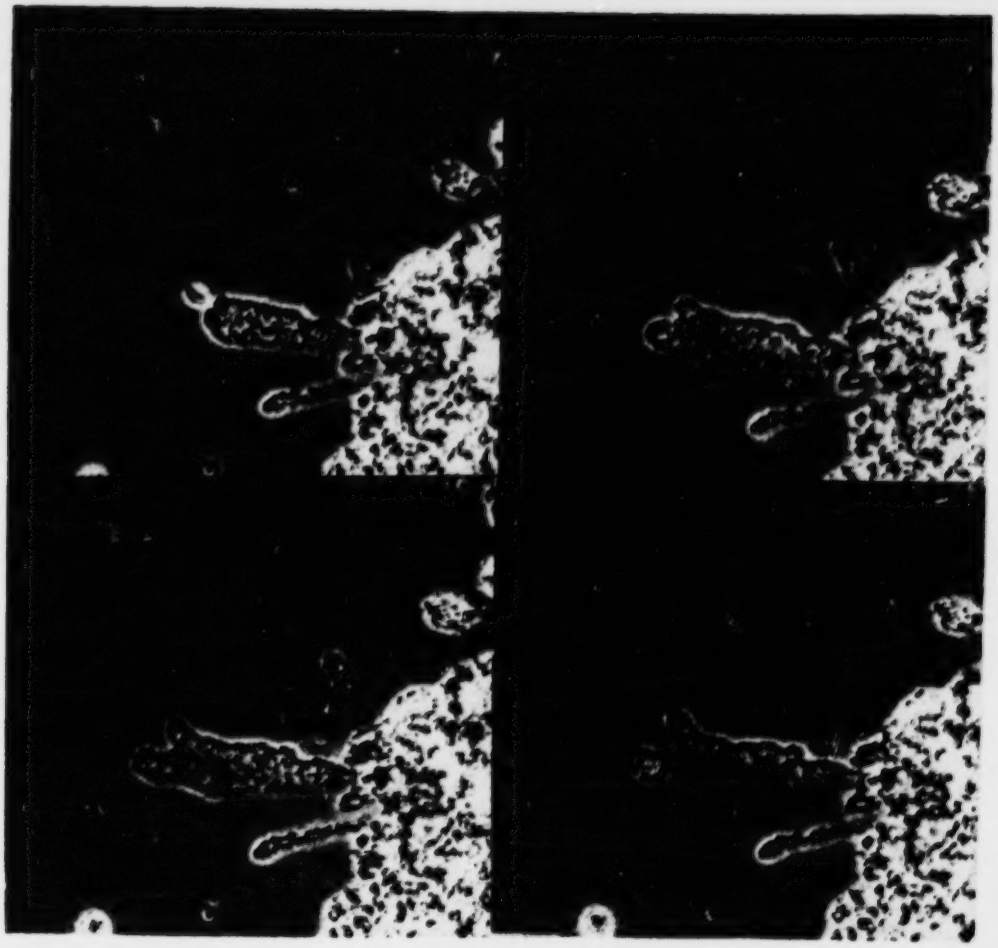

Fig. 8. Natural, Finger-Like, Bacterial Zoogloea Undergoing Branching. Note lateral branch of zoogloea (Z1) developed parallel to another elongating zoogloea (Z2) whereas zoogloea (Z3) did not increase in length during the entire 90 minute viewing period. Time elapsed (min): A, 0; B, 60; C, 75; D, 90. Phase contrast; wet mount. Bar equals 20 µm.

Fig. 9. Development of Natural, Finger-Like, Bacterial Zoogloeae from Activated Sludge Flocs. Note zoogloeae (Z) extend from flocs (F) towards interface which exists between entrapped air and water (arrow, far right). Phase contrast; wet mount. Field of view: 10^6 $\mu m.^2$

towards an interface formed between water and a small volume of air entrapped beneath the coverslip of the wet mount. The picture is suggestive of an aerotactic or chemotactic response by the bacterial zoogloeae. For unapparent reasons, bacterial zoogloeae may cease to continue extended growth in the early stages of development ultimately resulting in disintegration of the colony and dispersion of the bacteria (Fig. 10). The act may reflect a mechanism whereby the bacteria enter a swarm stage of behavior, ultimately resulting in the establishment of new zoogloea colonies through multiplication of the "swarmers". The factors stimulatory to the onset of a swarm stage were not determined but may entail conditions, e.g., nutrient deficiency, which are unfavorable for continued multiplication of the bacteria in the zoogloea.

Finger-like zoogloeae protruded from activated sludge flocs at a linear rate of extension (Fig. 11). Zoogloeae which grew outward from a single floc extended at similar rates whereas the development of zoogloeae from heterogeneous flocs often occurred at different extensions rates possibly indicating a sensitive effect of the microenvironment on the bacteria in the flocs or that the zoogloea-forming bacteria in the flocs differ from each other in some respects. The extensions rates of 30 finger-like zoogloeae were calculated from data obtained during microculture studies (Table 1). Seventy percent of those zoogloeae measured extended from activated sludge flocs at rates ranging from 5.1 to 15.0 μm per hr.

It was possible, by single frame analysis of the motion picture, to enumerate bacteria contained in certain developing, finger-like zoogloeae. Increases in cell numbers were found to be linear with time (Fig. 12) and the mean doubling time estimated for dividing cells seen in four zoogloeae was 2.0 hr. This generation time is remarkably similar to that obtained by axenic Zoogloea strains growing on CY medium in batch culture at 28 C (unpublished data).

SEROLOGY

Five of eight Zoogloea strains originally isolated by micromanipulation from natural finger-like zoogloeae fluoresced when stained with fluorescein labeled antiserum recovered from rabbits inoculated with cells of Z. ramigera 106. Only three of 34 Zoogloea strains isolated by streak plating wastewater samples of sodium m-toluate medium expressed a similar reaction. A slight antigen-antibody reaction took place between the labeled serum and Z. ramigera 115, however, further refinement of the antiserum successfully eliminated the cross reaction. Purification of the antiserum did not alter the intense fluorescence which occurred in reaction with slide smears of directly isolated Zoogloea strains. No cross reaction took place between the antiserum and Z. ramigera I-16-M, Streptococcus faecalis, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, P. fluorescens, and P. putida. In addition, serological tests were conducted on 71 axenic cultures of unidentified bacteria obtained from

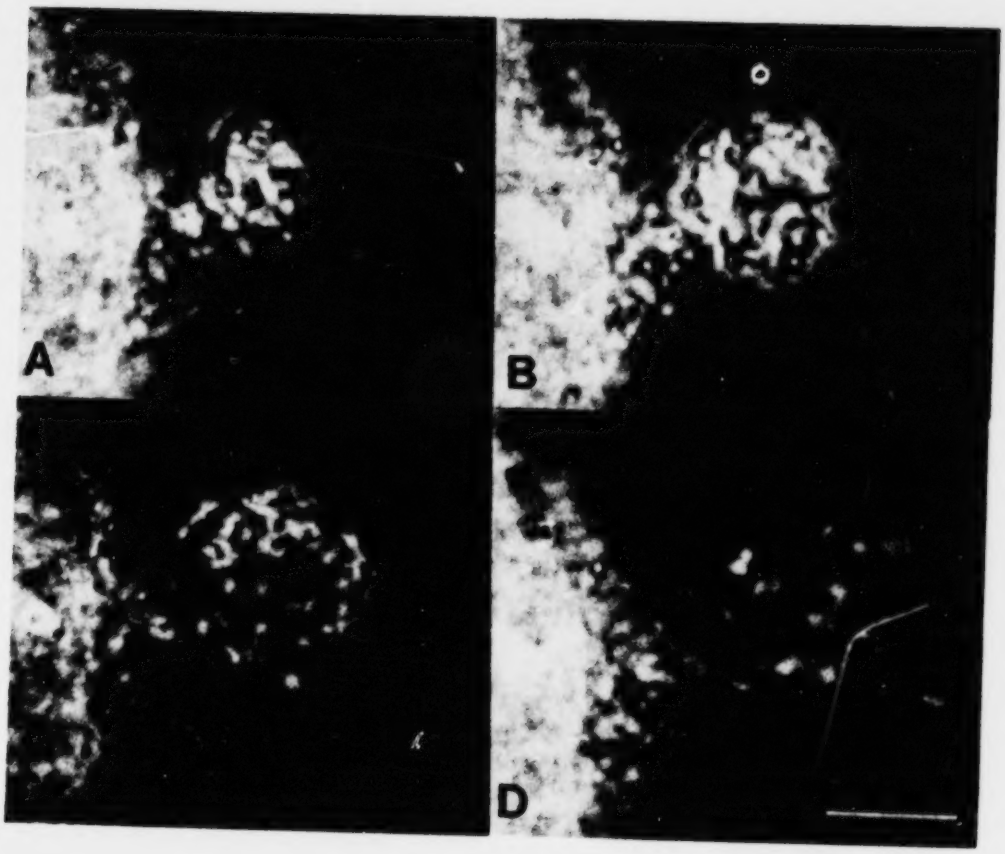

Fig. 10. Disintegration of a Natural, Bacterial Zoogloea. Time elapsed (min): A, 0; B, 67; C, 166; D, 210. Phase contrast; wet mount. Bar equals 10 µm.

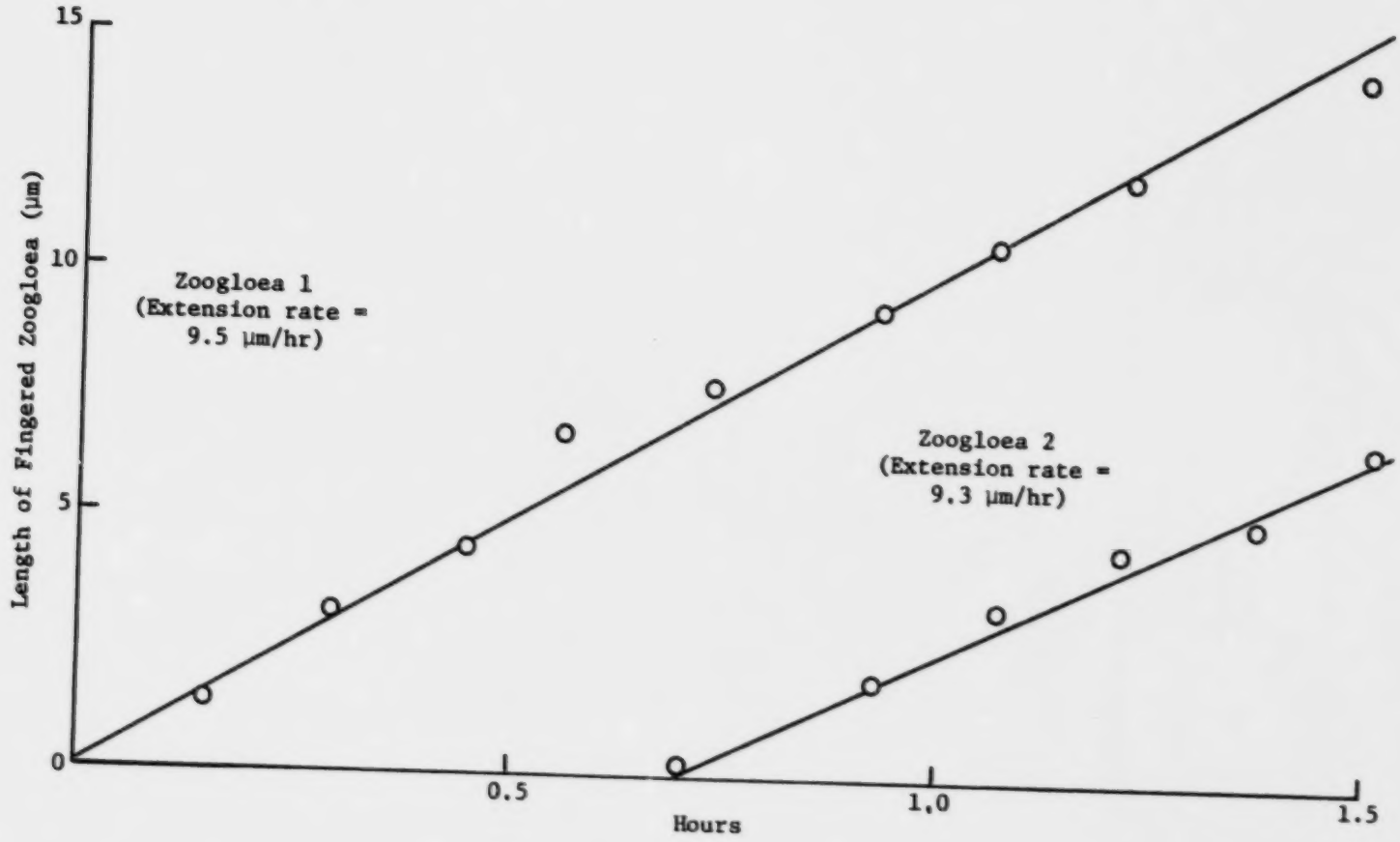

Fig. 11. Relationship Between Time and Extension of Two, Finger-Like Bacterial Zoogloeae from an Activated Sludge Floc.

Table 1. Extension of Bacterial Zoogloeae from Activated Sludge Flocs[a]

Extension rate, μm/hr (Class intervals)	Number of zoogloeae observed in each class interval	Percentage of zoogloeae in each class interval
0 - 5.0	2	7
5.1 - 10.0	13	43
10.1 - 15.0	8	27
15.1 - 20.0	5	17
20.1 - 25.0	0	0
25.1 - 30.0	1	3
30.1 - 35.0	0	0
35.1 - 40.0	1	3

[a] Total number of zoogloeae observed: 30.

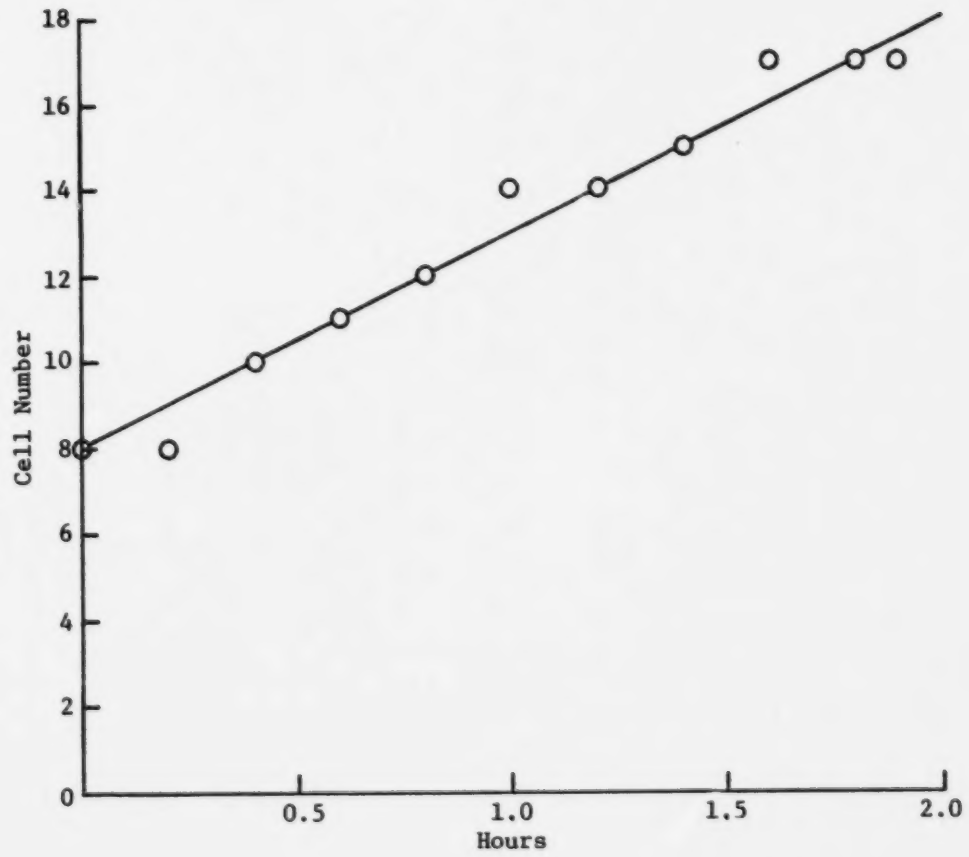

Fig. 12. Increase in Number of Bacteria During Extension of a Natural Finger-Like, Bacterial Zoogloea from an Activated Sludge Floc.

mixed liquor and scums formed at the surface of mixed liquors stored in glass beakers. These bacteria were isolated on CGY and activated sludge extract media and included 59 gram negative rods, 9 gram positive rods, and 3 gram positive cocci. None of the 71 unidentified cultures exhibited cross reaction with Z. ramigera 106 labeled antiserum (Table 2).

The purified Z. ramigera 106 labeled antiserum was seen to react with certain natural, bacterial zoogloeae obtained from the scums of stored mixed liquors. The affinity of the serum for the zoogloeae was apparent when treated smears were examined by tungsten (Fig. 13A) and ultraviolet light (Fig. 13B). Only bacterial cells which reacted with the antiserum were visible by ultraviolet fluorescence whereas many types of cells were seen in and around the zoogloeae under tungsten light. In another series of exposures with labeled serum-treated specimens, filamentous bacteria and the bacteria of finger-like zoogloeae were visible by tungsten light (Fig. 14A), however, only the cells within zoogloeae were apparent by ultraviolet illumination (Fig. 14B). Although most of the cells in certain natural finger-like zoogloeae reacted with the antiserium, relatively few of these were seen in serum treated activated sludge flocs (Fig. 15). However, it may be that the serum failed to effectively penetrate the flocs and make contact with many susceptible cells. In certain cases, activated sludge flocs which did not initiate formation of finger-like zoogloeae did contain single cells and small clumps of cells which reacted with the labeled antiserum (Fig. 16).

Certain natural, finger-like zoogloeae did not react with Z. ramigera 106 labeled antiserum. However, it was noted that when antiserum positively reacted with a finger-like zoogloea, all visible cells in the zoogloea were involved in the reaction. In contrast, zoogloeae not reactive with labeled antiserum contained no fluorescing cells by ultraviolet light. There were a large number of zoogloeae which did not react with the labeled antiserum. For example, in slide tests with zoogloeae developed in scums formed at the surface of mixed liquors collected from the State College and University Park wastewater treatment plants, only 30 and 54 percent, respectively, of the zoogloeae counted demonstrated a reaction with Z. ramigera 106 labeled antiserum (Table 3).

ZOOGLOEAL MATRIX (EXOPOLYMER)

Exopolymer of Zoogloea MP6 was found to be soluble in 0.1 N NaOH but not in 0.1 N HCl or lipid solvents (chloroform, acetone, and ethanol). Bacterial cells of zoogloeae which had been treated with 0.1 N NaOH appeared largely distorted and it was feared that the intracellular contents of damaged bacteria might seriously contaminate the exopolymer. Therefore, milder polymer recovery methods were desired and it was found that mechanical blending of zoogloeae in 0.02 M K_2HPO_4 or distilled water freed portions of the polymer while leaving the bacteria unaffected (Table 4).

Table 2. Specificity of Zoogloea ramigera 106 Antiserum in Fluorescent Antibody Tests with Axenic Bacteria

Bacteria	Number of strains	Number of strains showing intense fluorescence	Percent of strains showing intense fluorescence
Zoogloea strains (direct isolates)[a]	8	5	63
Zoogloea strains (indirect isolates)[b]	34	3	9
Zoogloea ramigera 115	1	0[c]	0
Zoogloea ramigera I-16-M	1	0	0
Streptococcus faecalis	1	0	0
Staphlococcus aureus	1	0	0
Escherichia coli	1	0	0
Proteus vulgaris	1	0	0
Pseudomonas aeruginosa	3	0	0
Pseudomonas fluorescens	1	0	0
Pseudomonas putida	1	0	0
Unidentified isolates[d]	71	0	0

[a] Zoogloea strains isolated by Unz and Dondero (65).

[b] Zoogloea strains isolated by Unz and Farrah (68).

[c] Slight initial fluorescence was observed which was eliminated by absorbing conjugated Zoogloea ramigera 106 antiserum with Z. ramigera 115.

[d] Bacteria isolated from mixed liquor and the scum formed at the surface of mixed liquor stored in beakers at 28C for 48 hrs.

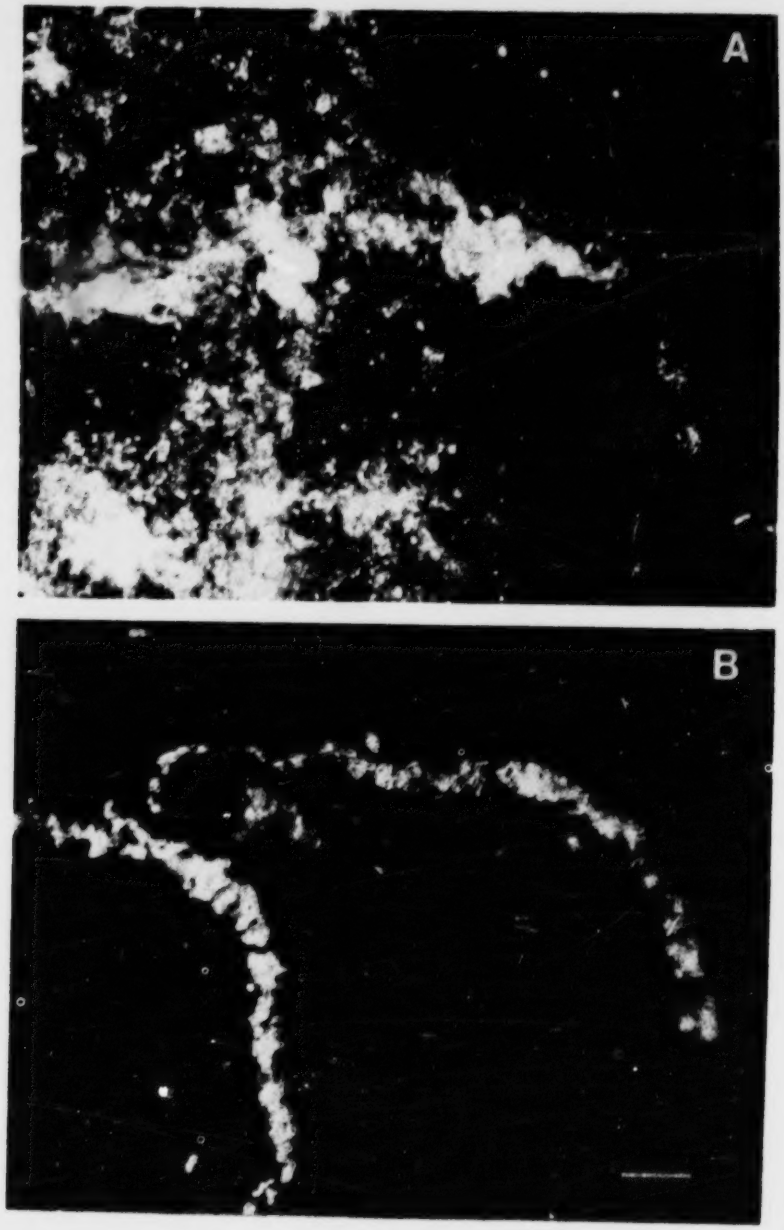

Fig. 13. Natural, Finger-Like, Bacterial Zoogloeae Treated with Zoogloea ramigera 106 Conjugated Antiserum. Specimen obtained from scum layer which developed on the surface of mixed liquor stored in a beaker; 48 hr, 28 C. Darkfield condenser with: A, tungsten light; B, ultraviolet light. Bar equals 10 µm.

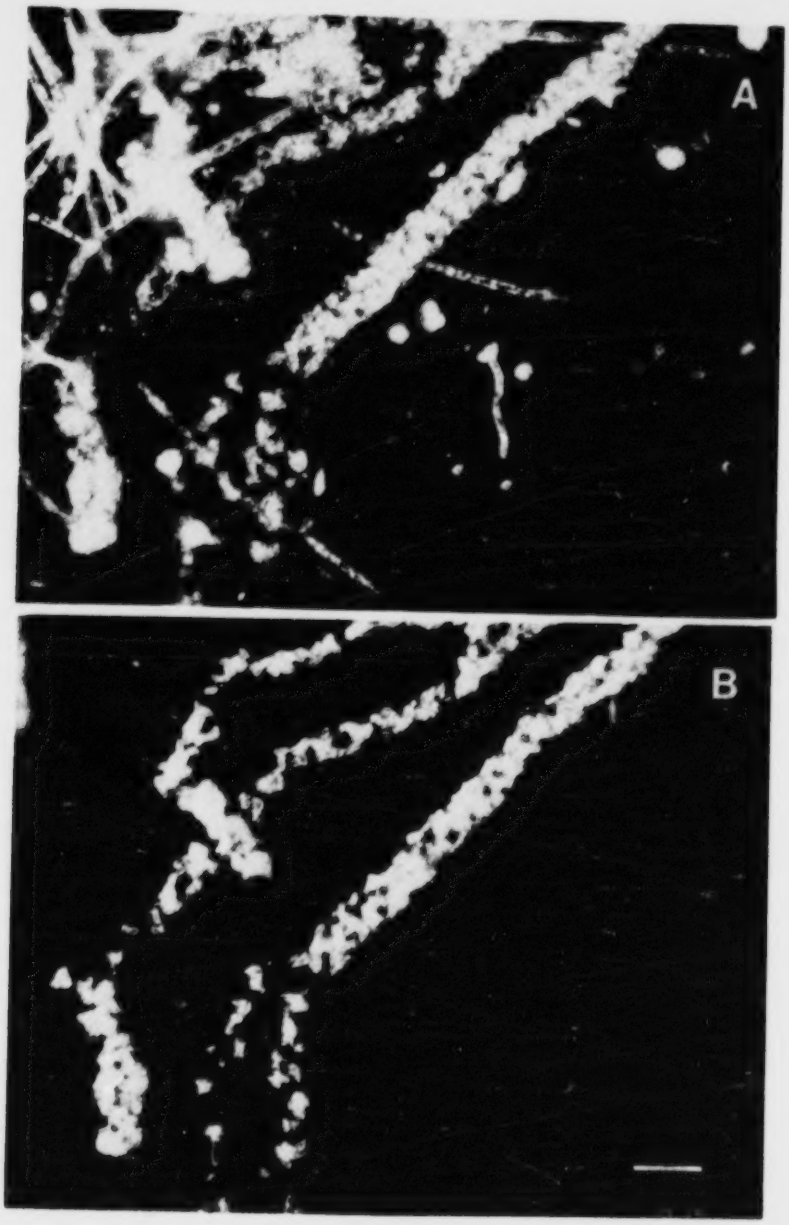

Fig. 14. Natural, Finger-Like, Bacterial Zoogloeae and Filamentous Bacteria Treated with Zoogloea ramigera 106 Conjugated Antiserum. Specimen obtained from scum layer which developed on the surface of mixed liquor stored in a beaker; 48 hr, 28 C. Darkfield condenser with: A, tungsten light; B, ultraviolet light. Bar equals 10 µm.

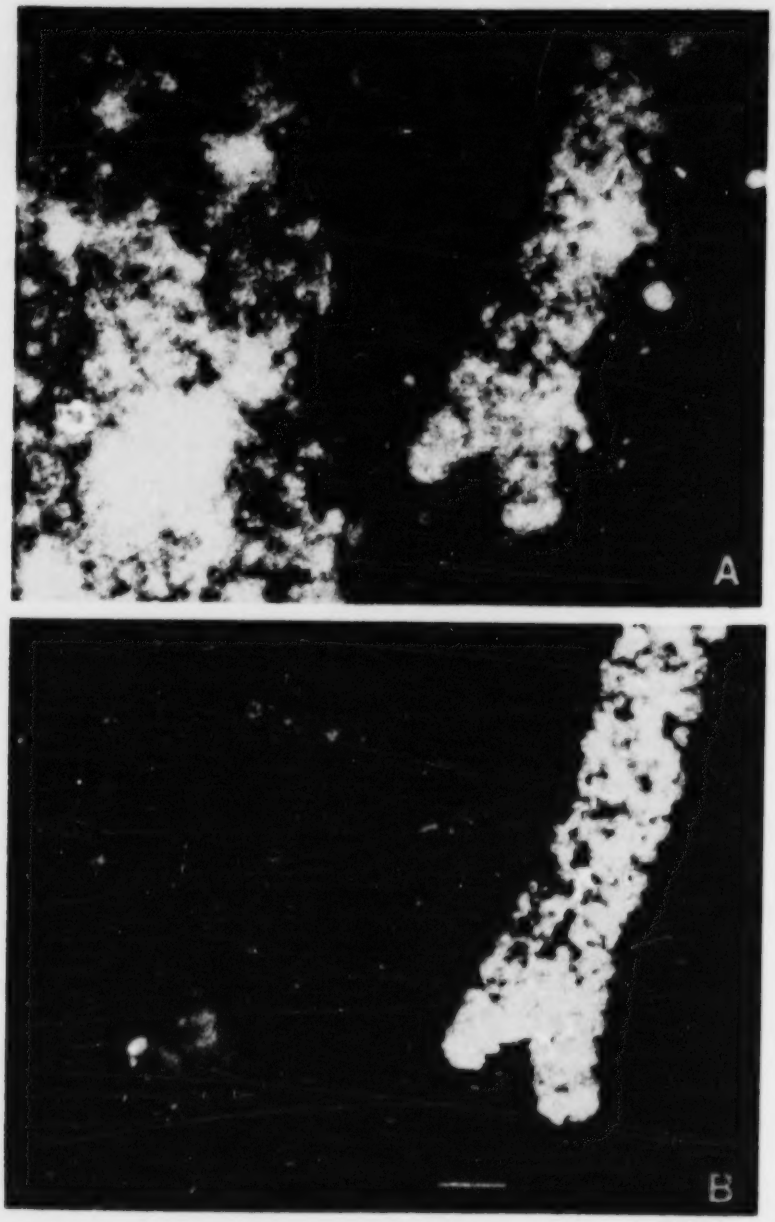

Fig. 15. Bifurcate, Finger-Like, Bacterial Zoogloea and an Activated Sludge Floc Treated with Zoogloea ramigera 106 Conjugated Antiserum. Specimen obtained from scum layer which developed on the surface of mixed liquor stored in a beaker; 72 hr, 28 C. Darkfield condenser with: A, tungsten light; B, ultraviolet light. Bar equals 10 µm.

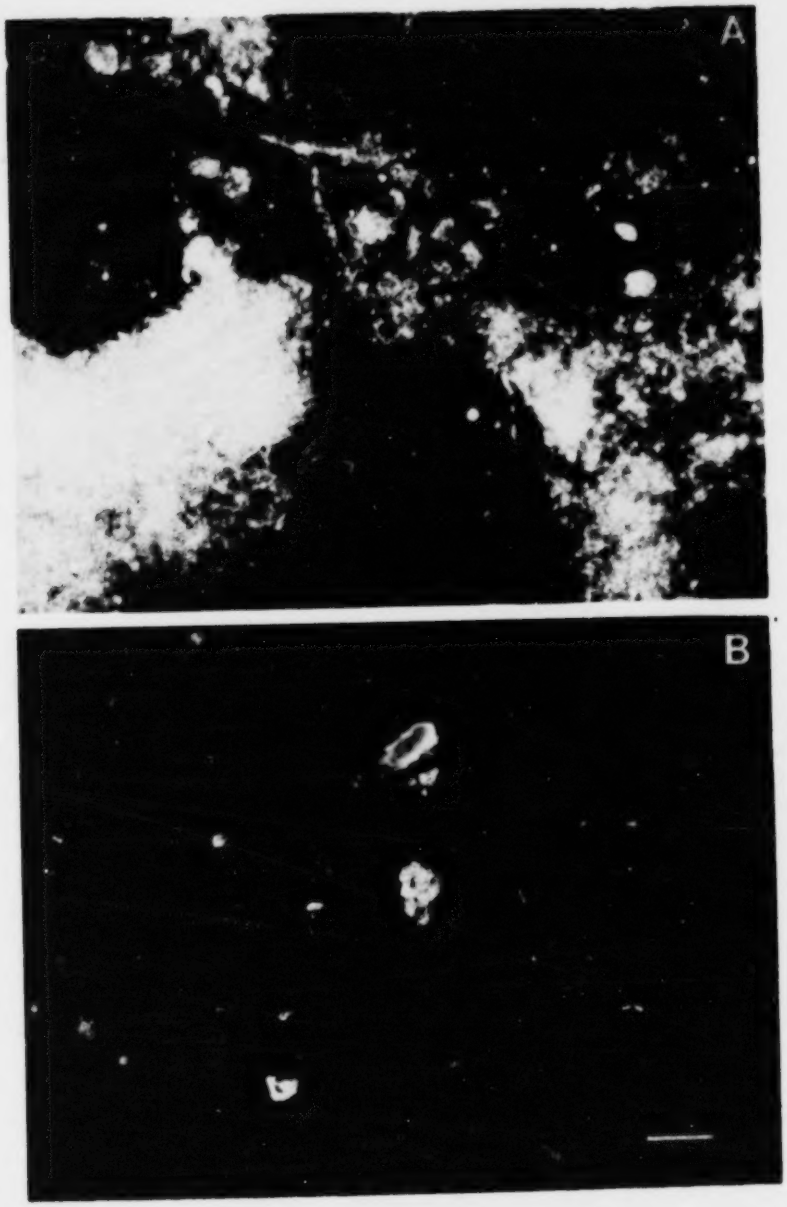

Fig. 16. Activated Sludge Flocs Treated with Zoogloea ramigera 106 Conjugated Antiserum. Brightly fluorescing individual cells and cell aggregates, presumably Zoogloea sp., are distinguishable in flocs. Specimen obtained from scum layer which developed on the surface of mixed liquor stored in a beaker; 72 hr, 28 C. Darkfield condenser with: A, tungsten light; B, ultraviolet light. Bar equals 10 µm.

Table 3. Specificity of Zoogloea ramigera 106 Antiserum in Fluorescent Antibody Tests with Natural, Finger-like Zoogloeae Present in Mixed Liquor Scum[a]

Source of mixed liquor	Number of finger-like zoogloeae observed	Number of finger-like zoogloeae showing fluorescence	Percentage of finger-like zoogloeae showing fluorescence
State College, Pa., wastewater treatment plant	23	7	30
University Park, Pa., wastewater treatment plant	110	54	49

[a] Scums formed at the surface of mixed liquor stored in beakers in 48 hrs at 28C.

Table 4. Total and Viable Numbers of Bacteria Released from Zoogloea MP6 Zoogloeae with Dipotassium Phosphate[a]

Molar concentration of dipotassium phosphate	Viable cell count per ml x 10^{-7} [b]	Total cell count per ml x 10^{-7}
0.00	42	115
0.02	42	105
0.05	21	66
0.10	0.15	34

[a] Zoogloeae blended for 1 min in solutions; final pH 10.

[b] Mean of duplicate cell counts.

Polymer obtained from zoogloeae blended in 0.02 M K_2HPO_4 was soluble and could be concentrated to form a clear, viscous solution. In contrast, zoogloeae blended in distilled water released polymer which, upon concentration, produced a cloudy, viscous liquid. Exopolymer suspended in water was cell-free and intact. Zoogloeae of Zoogloea MP6 and cell-free isolated polymer are shown in Figures 17 and 18.

The efficiency of different treatment methods for polymer recovery varied. Although blending zoogloeae in water or 0.02 M K_2HPO_4 did not visibly affect the viability of bacterial cells, less than half of the available exopolymer could be recovered(Table 5). Boiling zoogloeae in 0.02 M K_2HPO_4 or approx. 0.001 N NaOH (both solutions having a pH of 10) allowed separation of most of the exopolymer. For routine analytical work, exopolymer was obtained by boiling zoogloeae suspensions in 0.02 M K_2HPO_4 for 10 min at pH 10. No amino sugar-containing exopolymers could be obtained from flocs of Z. ramigera 115 or Z. ramigera I-16-M using the aforementioned procedure and the failure to detect amino sugar in concentrates was considered proof that amino sugars were not being released from cell walls during treatment (Table 6).

Purified and dissolved Zoogloea MP6 expolymer revealed no absorption peaks at 260 nm or 280 nm indicating the absence of nucleic acids and protein (Fig. 19). Hydrolyzed Zoogloea MP6 exopolymer was found by paper chromatography to consist of two major and one minor chemical components all of which were reducing and ninhydrin positive (Fig. 20). In an attempt to identify these compounds, several known reference chemicals were chromatographed with the hydrolyzed sample (Fig.21). One unknown substance (spot B) co-chromatographed with D-glucosamine · HCl. Unknown compound (spot A) migrated similarily to D-glucose, however, it was unlike glucose in that it gave a positive ninhydrin reaction. Unknown compound (spot C) was immobile relative to reference and other unknown chemical components.

Purified Z. ramigera 106 exopolymer was found by paper chromatography to be similar in chemical constituents to the polymer of Zoogloea MP6. Culturing the Zoogloea strains on media of different chemical composition did not appear to affect the chemical quality of the exopolymer produced (Table 7).

Evidence of amino sugars in the exopolymers of Zoogloea strains was of interest regarding the possibility of indirectly quantifying polymer production by analyzing for amino sugars. The optimum hydrolysis time for liberation of the amino sugar from exopolymer with boiling aqueous 6 N HCl was found to be 0.75 hr (Table 8). A longer heating period did not produce an appreciable increase in free amino sugar.

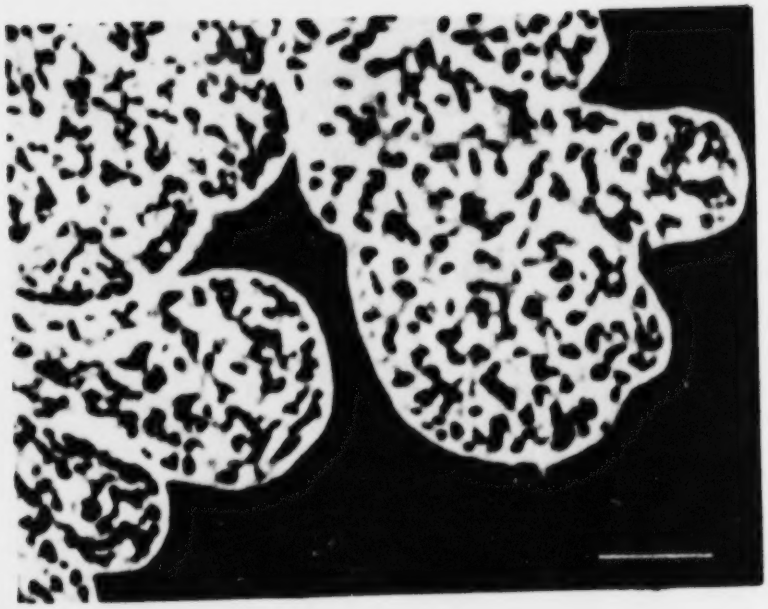

Fig. 17. Zoogloeae of Zoogloea MP6. Zoogloeae treated with India ink to accentuate matrix. Static culture, sodium lactate-mineral salts medium; 48 hr, 28 C. Phase contrast; wet mount. Bar equals 10 µm.

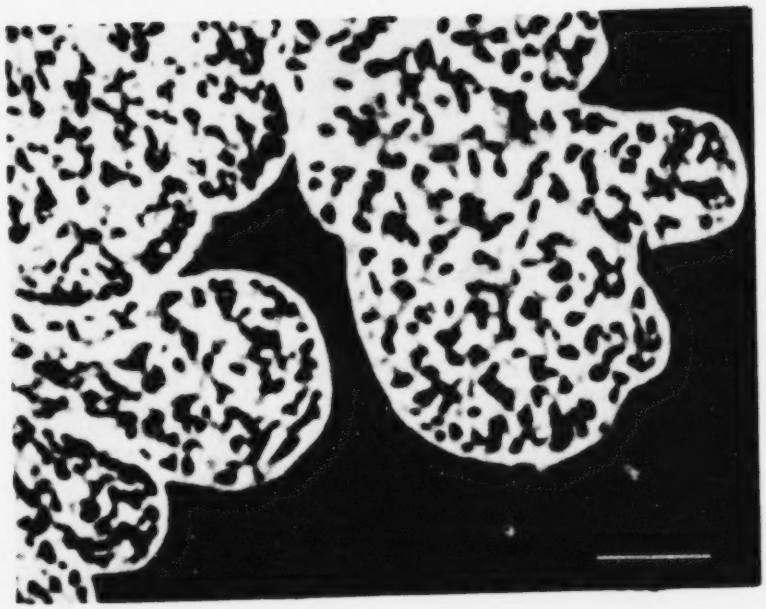

Fig. 17. Zoogloeae of Zoogloea MP6. Zoogloeae treated with India ink to accentuate matrix. Static culture, sodium lactate-mineral salts medium; 48 hr, 28 C. Phase contrast; wet mount. Bar equals 10 μm.

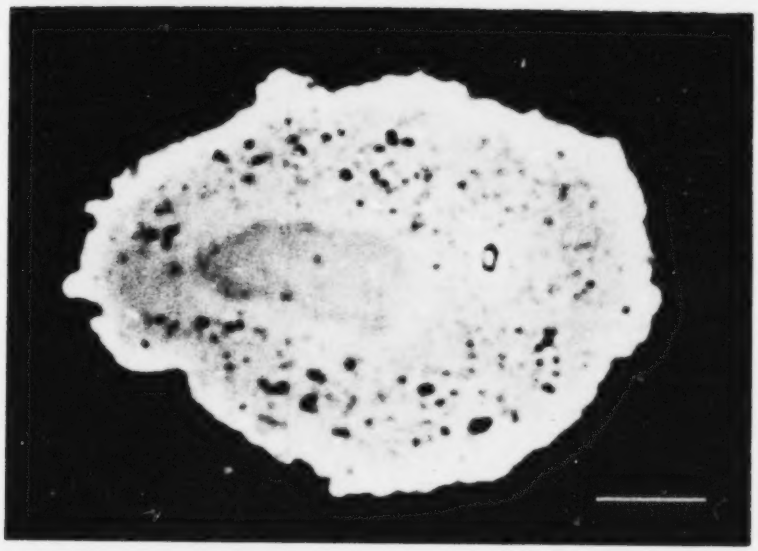

Fig. 18. Water-Sheared, Cell-Free Exopolymer of Zoogloea MP6. Exopolymer treated with India ink to accentuate boundary. Phase contrast; wet mount. Bar equals 10 μm.

Table 5. Separation and Recovery of Exopolymer from Zoogloea MP6.

Exopolymer extraction method	Amino sugar in exopolymer recovered (µg/mg dry weight of culture)	Amino sugar in unrecovered exopolymer (µg/mg dry weight of culture)[a]
Blending with 0.02 M K_2HPO_4, pH 10	35	45
Blending with distilled water	30	55
Boiling with 0.02 M K_2HPO_4, pH 10	70	10
Boiling with distilled water adjusted to pH 10 with NaOH	70	15
Control (untreated sample)	0	90

[a] Unseparated exopolymer together with bacterial cells was hydrolyzed in 6 N HCl for 45 minute in a boiling water bath. Insoluble residues were removed by centrifugation before the clear supernatant was dried to remove HCl and analyzed for amino sugar.

Table 6. Separation of Recovery of Exopolymer from Zoogloea ramigera 115 and Z. ramigera I-16-M

Zoogloea strain	Exopolymer recovery method[a]	Amino sugar in exopolymer recovered, µg/mg dry weight of culture	Reducing substances in exopolymer recovered, µg/mg dry weight of culture
115	Boiling for 10 min	0	1
115	Blending for 1 min	0	0.5
I-16-M	Boiling for 10 min	less than 0.1	0.5
I-16-M	Blending for 1 min	less than 0.1	0.3

[a] Zoogloeae suspended in 0.02 M K_2HPO_4 at pH 10.

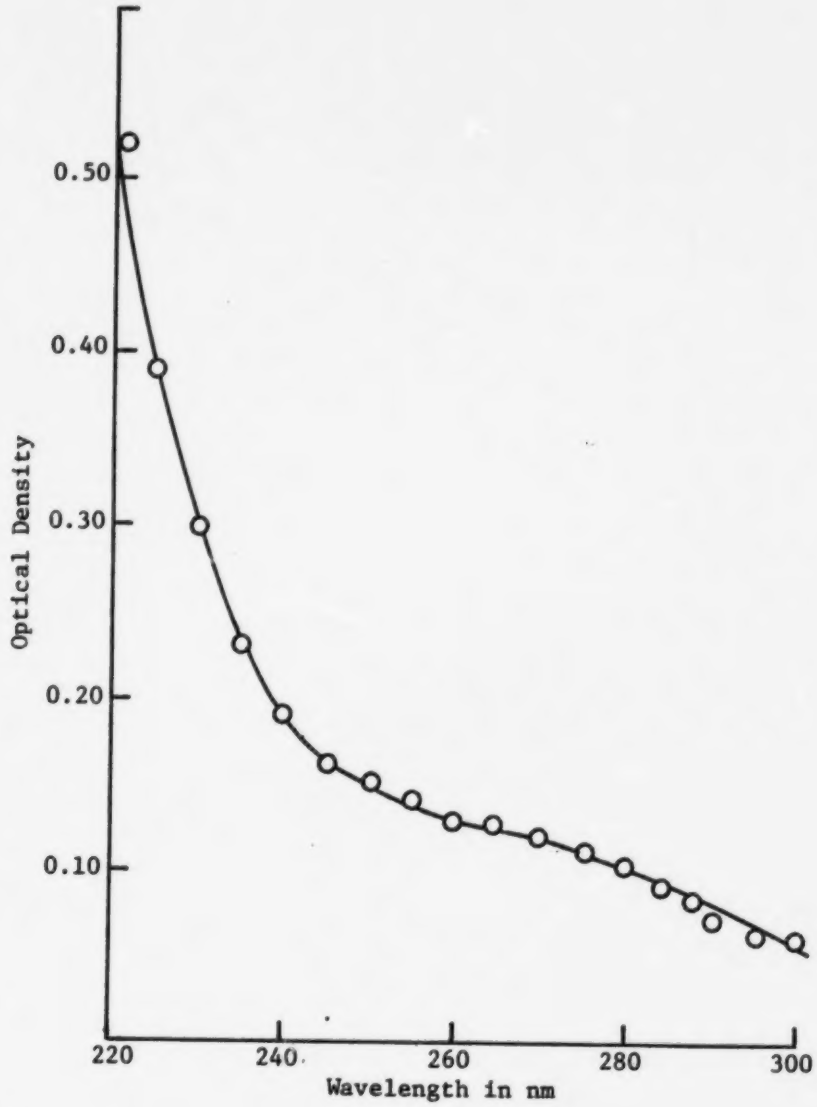

Fig. 19. Absorption Spectrum of Unhydrolyzed Zoogloea MP6 Exopolymer.

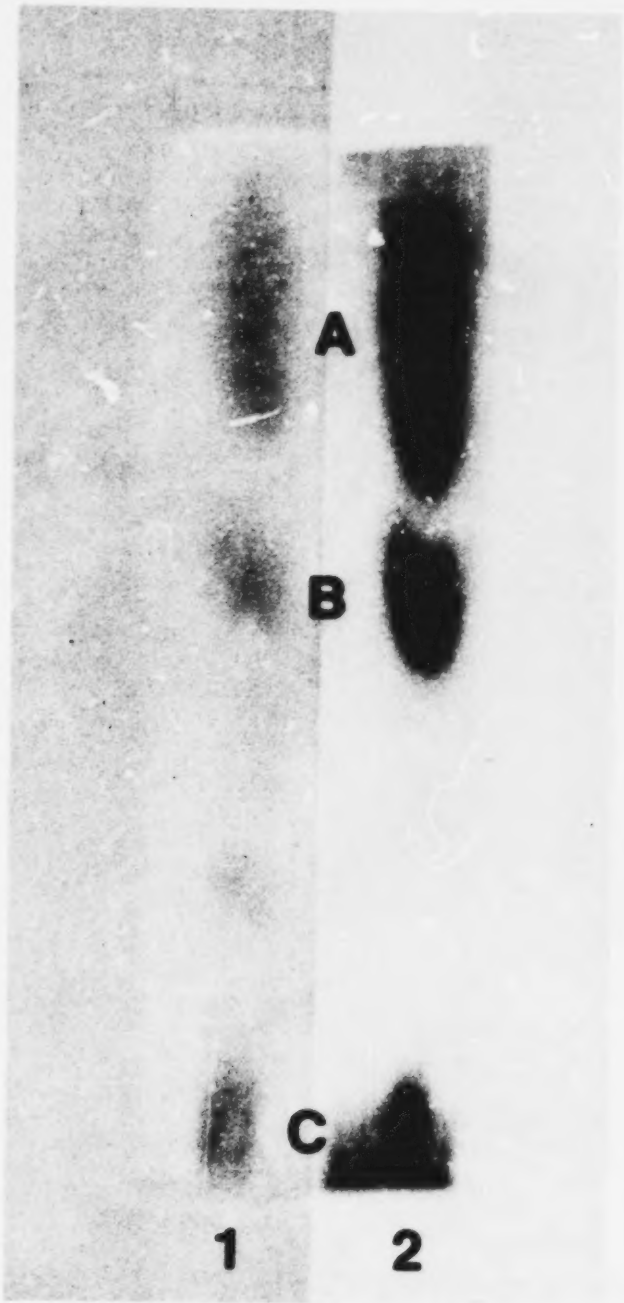

Fig. 20. Comparative Paper Chromatography of Acid Hydrolyzed Zoogloea MP6 Exopolymer Revealing Spots A, B, and C. Isopropanol-water (4:1) solvent system. Chromatogram number 1 processed with ninhydrin. Chromatogram number 2 processed with silver nitrate reagent.

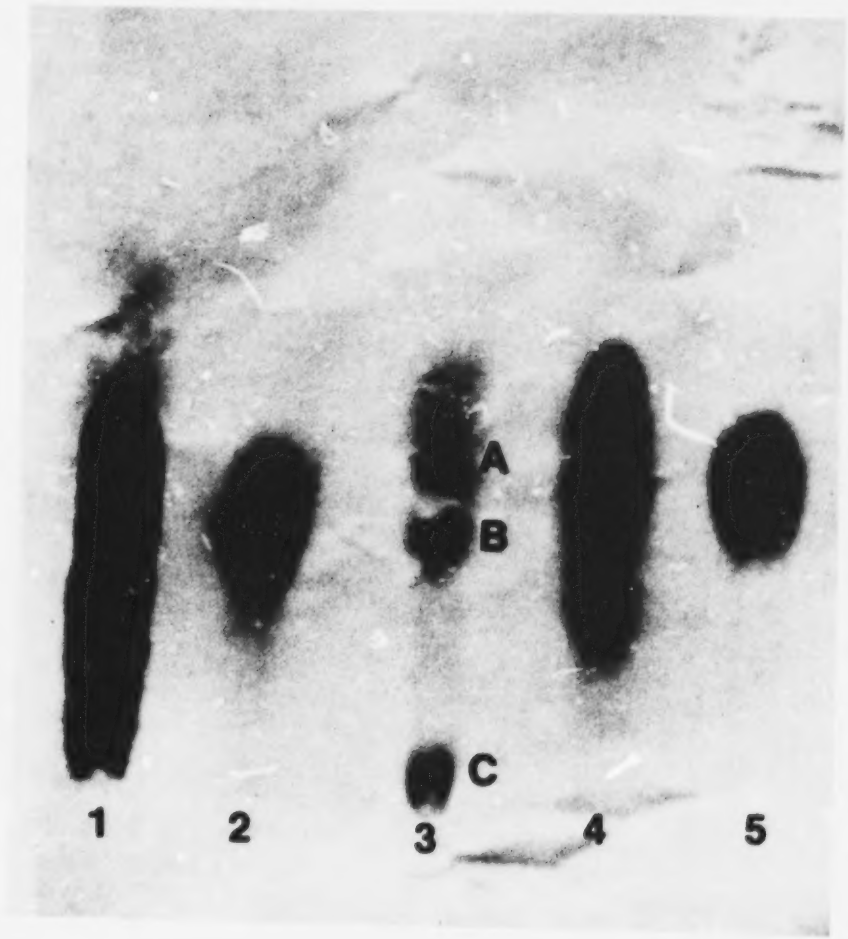

Fig. 21. Paper Chromatography of Acid Hydrolyzed Zoogloea MP6 Exopolymer and Reference Compounds. Isopropanol-water (4:1) solvent system. 1, D-glucuronic acid; 2, D-glucosamine·HCl; 3, exopolymer hydrolysate; 4, muramic acid·HCl; 5, D-glucose. Chromatogram processed with silver nitrate reagent.

Table 7. Paper Chromatography of Acid Hydrolyzed Exopolymer Obtained from Zoogloea ramigera 106 and Zoogloea MP6[a]

Zoogloea Strain number	Culture medium	$R_{D\text{-glucosamine} \cdot HCl}$ values of exopolymer components		
		Spot A	Spot B	Spot C
106	Basal medium + sodium lactate	1.25	1.00	0.07
MP6	Basal medium + sodium lactate	1.27	1.00	0.14
MP6	Casitone - yeast autolysate	1.40	1.08	0.07
MP6	Trypticase Soy	1.30	1.03	0.07

[a] Solvent system: isopropanol - water (4:1)

Table 8. Free Amino Sugar Content of Zoogloea MP6 and Zoogloea ramigera 106 Exopolymers Hydrolyzed with 6 N HCl in Boiling Water

Hydrolysis period (hr)	Percent of maximum amino sugar released from exopolymer	
	Strain MP6	Strain 106
0.25	91	100
0.75	95	96
2.0	100	96
6.0	91	94
12.0	91	89

Elution of the major chemical components from hydrolyzed Zoogloea MP6 exopolymer was accomplished by column separation. However, only two substances were recovered having R glucosamine values of 0.95 and 1.77, respectively (Fig. 22). The substance designated spot C on paper chromatograms traveled very poorly and was not recovered. In all probability, the compound which appeared first in the column effluent was glucosamine and corresponds to spot B on paper chromatograms. The second amino sugar to leave the column may have been fucosamine although no reference standard was available for comparison.

Hexosamines were not found to exist as the N-acetyl form in crude Zoogloea MP6 zoogloeae or in purified and hydrolyzed Zoogloea MP6 exopolymer or water suspensions of cell free exopolymer (Table 9). However, amino sugars were present in all of these samples.

Quantitative analyses of Zoogloea MP6 exopolymer revealed the dry weight of acid hydrolyzable fraction to be 69 percent of polymer dry weight with reducing substances and amino sugars accounting for approximately 19 and 16-18 percent, respectively, of the polymer dry weight. Hexose, uronic acids, and ether soluble substances constituted approximately one percent of the dry weight of polymer (Table 10).

Purified and unhydrolyzed Zoogloea MP6 exopolymer was titrated with 0.01 N NaOH and an equivalent weight of approximately 1500 was obtained which corresponds to one acidic residue per 10 carbohydrate residues.

The exopolymers of natural zoogloeae and Zoogloea MP6 were not observed to be microfibrilar by electron microscopy.

Chemical analyses of acid hydrolyzed activated sludge exopolymer revealed hexose and amino sugars but no uronic acids. Amino sugars were the major portion of the reducing substances liberated (Table 11). However, the percentage of amino sugars and reducing substances liberated were much lower than that found upon analysis of the exopolymer of Zoogloea MP6. Paper chromatography of the acid hydrolyzed activated sludge exopolymer revealed two major reducing spots, one of which could be identified as glucosamine. On this basis, activated sludge polymer bears some resemblance to the exopolymer of Zoogloea MP6, however, column chromatography and fractionation of the sludge polymer is needed to provide a more meaningful analysis of the chemical composition.

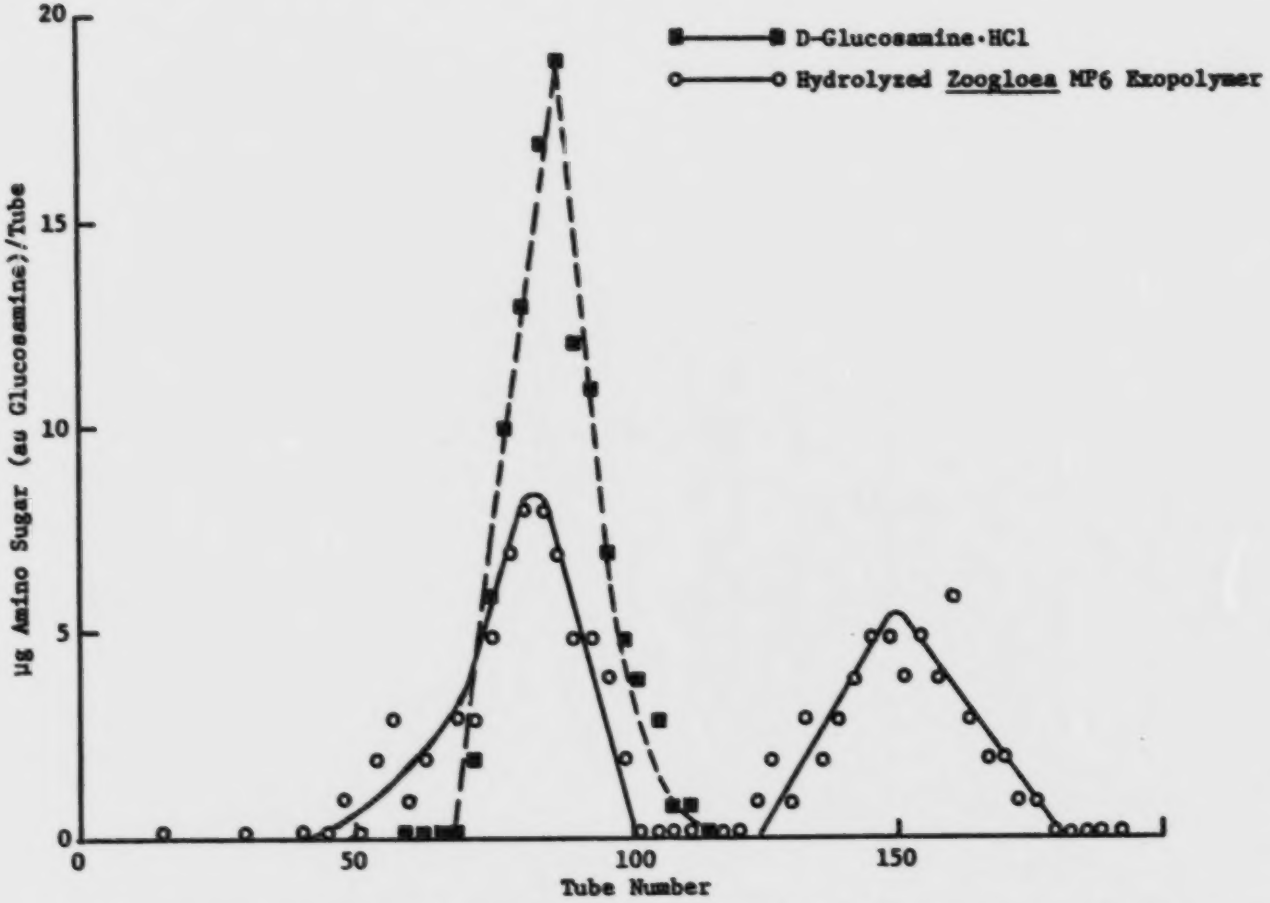

Fig. 22. Column Separation of Amino Sugars Present in Acid Hydrolyzed Zoogloea MP6 Exopolymer. Elution by 0.3 N HCl on Dowex 50 column.

Table 9. Absence of N-Acetyl Hexosamines in Exopolymer of Zoogloea MP6

Nature of samples	µg N-acetyl hexosamines (N-acetyl glucosamine used as standard)	µg amino sugar liberated from acid hydrolyzed samples[b]
Suspension of zoogloeae	0	160
Exopolymer dissolved with K_2HPO_4[a]	0	40
Exopolymer removed with water[a]	0	115

[a] Samples homogenized for 1 minute in a Waring blender; cells removed by centrifugation at 27,000 x gfor 10 minutes

[b] Samples hydrolyzed with 6 N HCl for 45 minutes in a boiling water bath; HCl removed by drying samples in air.

Table 10. Chemical Composition of Zoogloea MP6 Exopolymer[a]

Analytical test	Reference compound	Weight of substance (mg) Run 1	Run 2	Percent of unhydrolyzed sample Run 1	Run 2
Dry weight, unhydrolyzed sample		3.2	1.0	100	100
Reducing substances	D-glucosamine·HCl	0.61	0.19	19	19
Amino sugar	D-glucosamine·HCl	0.53	0.18	16	18
Hexose	D-glucose	0.03	0.03	1	3
Uronic acid	D-glucuronic acid	0.03	0.01	1	1

[a] Cells cultured in sodium lactate - mineral salts medium; exopolymer removed from cells by blending with 0.02 M K_2HPO_4, pH 10, and purified by precipitation with cetyltrimethylammonium bromide.

Table 11. Chemical Composition of Activated Sludge Exopolymer

Sample	Analytical test	Weight of substance recovered (mg)	Percent by weight of unhydrolyzed sample
Unhydrolyzed polymer	Dry weight	78.0	100.0
Hydrolyzed polymer	Amino sugar	1.0	1.3
	Reducing substance	1.5	1.9
	Hexose	2.9	3.7
	Uronic acid	0.0	0.0

BACTERIAL FLOCCULATION

Growth and zoogloea formation by Zoogloea MP6

The growth cycle of Zoogloea MP6 cultured in sodium lactate-mineral salts medium is shown in Figure 23. An index of flocculation (I. F.) equal to the difference between optical density values of unsettled (homogenous) and 4-hr settled culture divided by the optical density value of the unsettled culture was used to compare the flocculation of organisms at different stages of the growth cycle and under different culture conditions. Large I. F. values were indicative of good bioflocculation and values of 0.20 and less were considered representative of non-flocculent cultures. Measurable flocculation of Zoogloea strains was found to begin in the late logarithmic phase of growth becoming maximum at the onset of the stationary phase. During early stages of flocculation, a few encapsulated cells were observed to form a nucleus to which other bacteria attached themselves (Fig. 24). Bacterial flocculation proceeds at a more rapid rate than amino sugar production although amino sugars continued to be synthesized and incorporated into exopolymer even after maximum bacterial flocculation was reached. Apparently, exopolymer production does not cease even when the net growth approaches zero.

Metal ions and flocculation

Metallic cations, including mono-, bi-, and tri-valent forms, had little effect on promoting flocculation of Zoogloea MP6 (Figs. 25 and 26). Zinc and manganese salts were toxic in concentrations of 0.01 mM and 1.0 mM, respectively. Only calcium salts in concentrations of 0.1 mM or greater appreciably influenced flocculation of the bacteria as evidenced by large I. F. values. However, calcium ion was not always effective as a flocculating agent for the Zoogloea strains tested. On one occasion, Z. ramigera 106 cells did not flocculate well in the absence or presence of calcium ion (Fig. 27). Judging from the results of the foregoing experiments, it appears that calcium ion merely serves to increase the efficiency of cell aggregation over that possible through natural bioflocculation and does not induce normally dispersed cells to coalesce. Index of flocculation values for Zoogloea MP6, 21, and Z. ramigera 106 cultured in the presence of various concentrations of metal ions are presented in Table 12. Both NaBr and NaF were tested at Na ion concentrations of 1.0 millimole per liter and produced no effect on flocculation. The variability experienced in the effect of calcium ion on flocculation of Zoogloea strains prompted further investigation into the nature of the test organisms. It was noted that the extent of flocculation varied from time to time with the same Zoogloea strain under similar culture conditions. Although cultures of well and poorly flocculated cells both formed surface films on standing, these pellicles did not resist agitation equally well. Mechanical shaking of the well flocculated culture disrupted the pellicle into small fragments although cells remained in zoogloeae and the supernatant was practically clear.

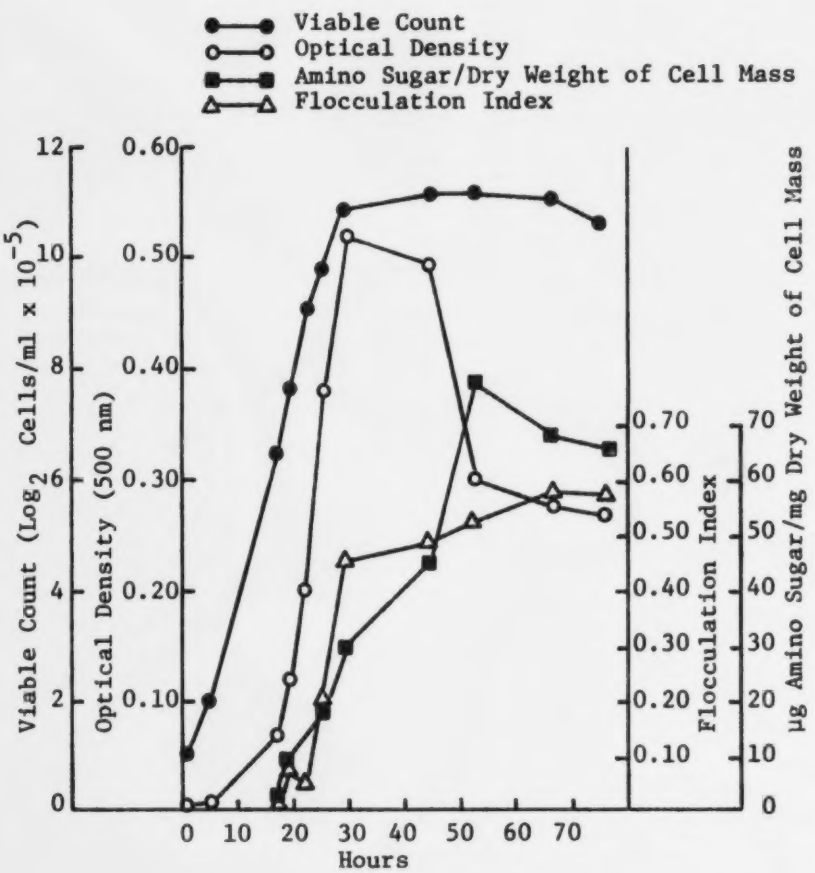
Viable Count
Optical Density
Amino Sugar/Dry Weight of Cell Mass
Flocculation Index
Viable Count (Log2 Cells/ml x 10-5)
Optical Density (500 nm)
Flocculation Index
µg Amino Sugar/mg Dry Weight of Cell Mass
Hours
0 10 20 30 40 50 60 70
0 2 4 6 8 10 12
0.10 0.20 0.30 0.40 0.50 0.60
0.10 0.20 0.30 0.40 0.50 0.60 0.70
0 10 20 30 40 50 60 70

Fig. 23. Growth, Flocculation, and Amino Sugar Production by Zoogloea MP6. Sodium lactate-mineral salts culture medium.

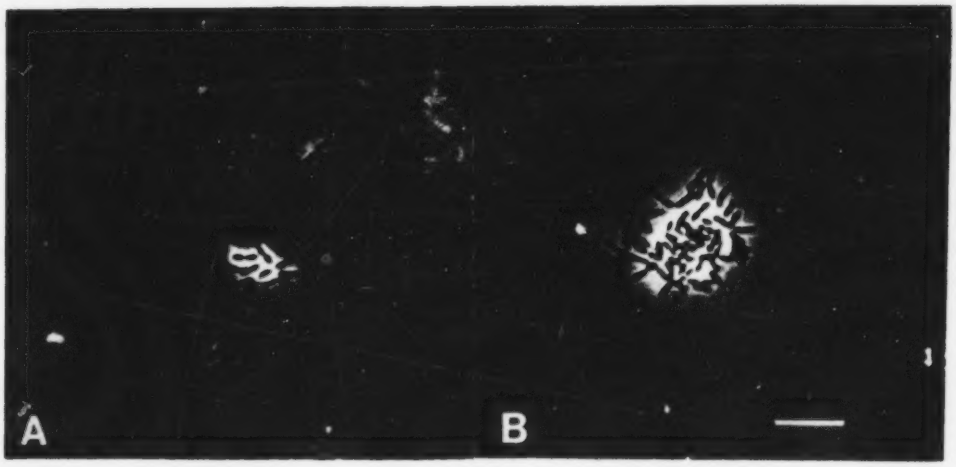

Fig. 24. Development of Zoogloeal Flocs by Zoogloea MP6. A, nucleus of zoogloea-forming cells in tiny floc; B, larger floc consisting of nucleus of zoogloea-forming cells to which many nonencapsulated bacteria are attached. Flocs treated with India ink to accentuate zoogloeal matrix boundary. Sodium lactate-mineral salts culture medium; 32 hr, 20 C. Phase contrast; wet mount. Bar equals 10 µm.

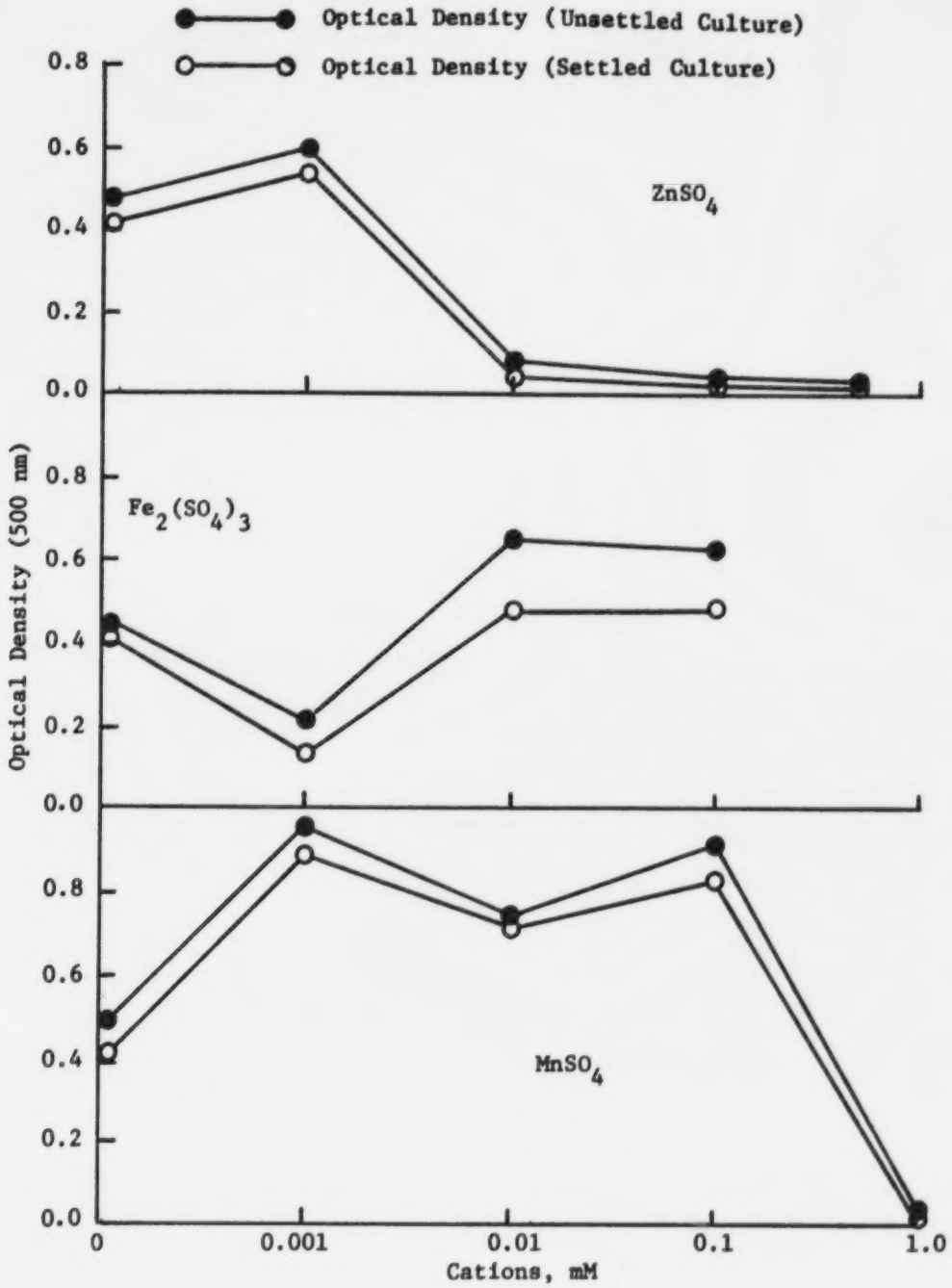

Fig. 25. Influence of Cations on Flocculation of Zoogloea MP6.

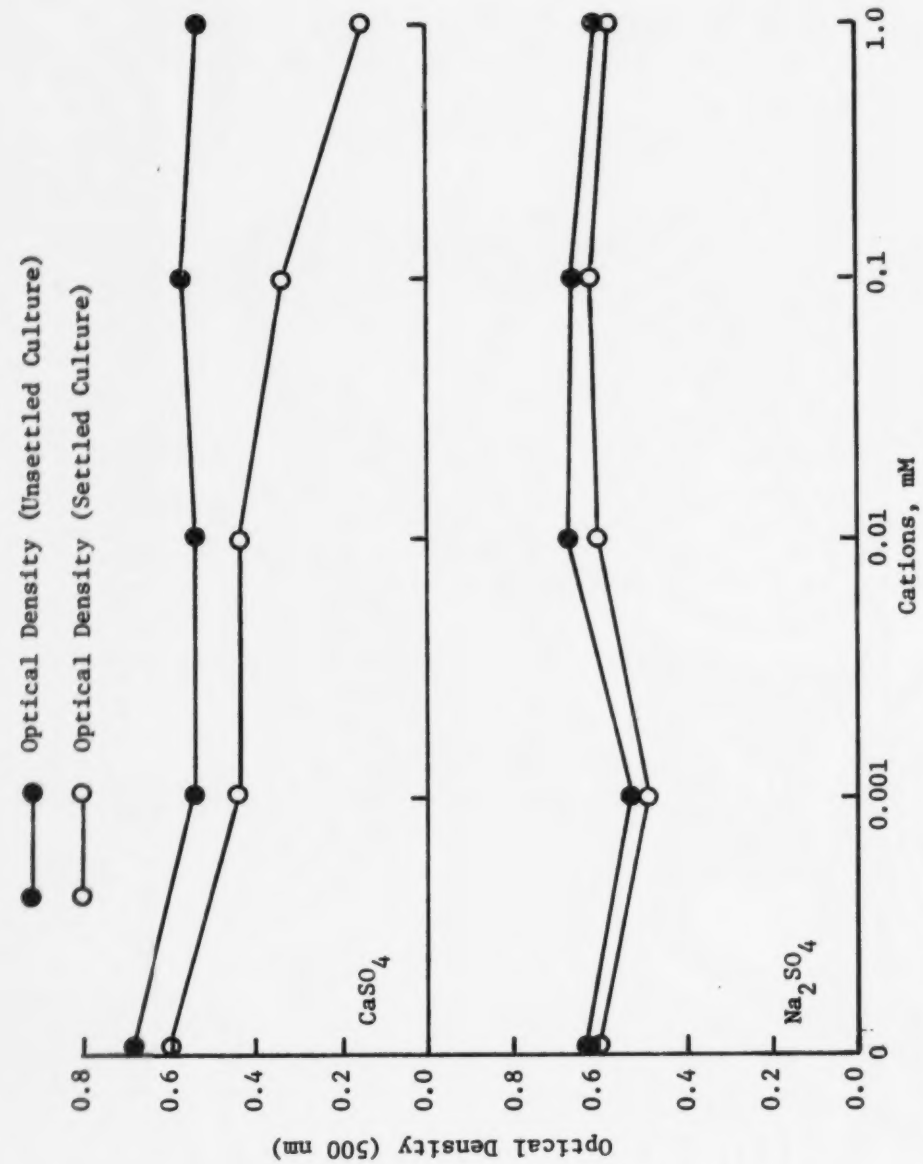
Optical Density (Unsettled Culture)
Optical Density (Settled Culture)
Optical Density (500 nm)
0.8
0.6
0.4
0.2
0.0
$CaSO_4$
Na_2SO_4
0
0.001
0.01
0.1
1.0
Cations, mM

Fig. 25. (Cont'd)

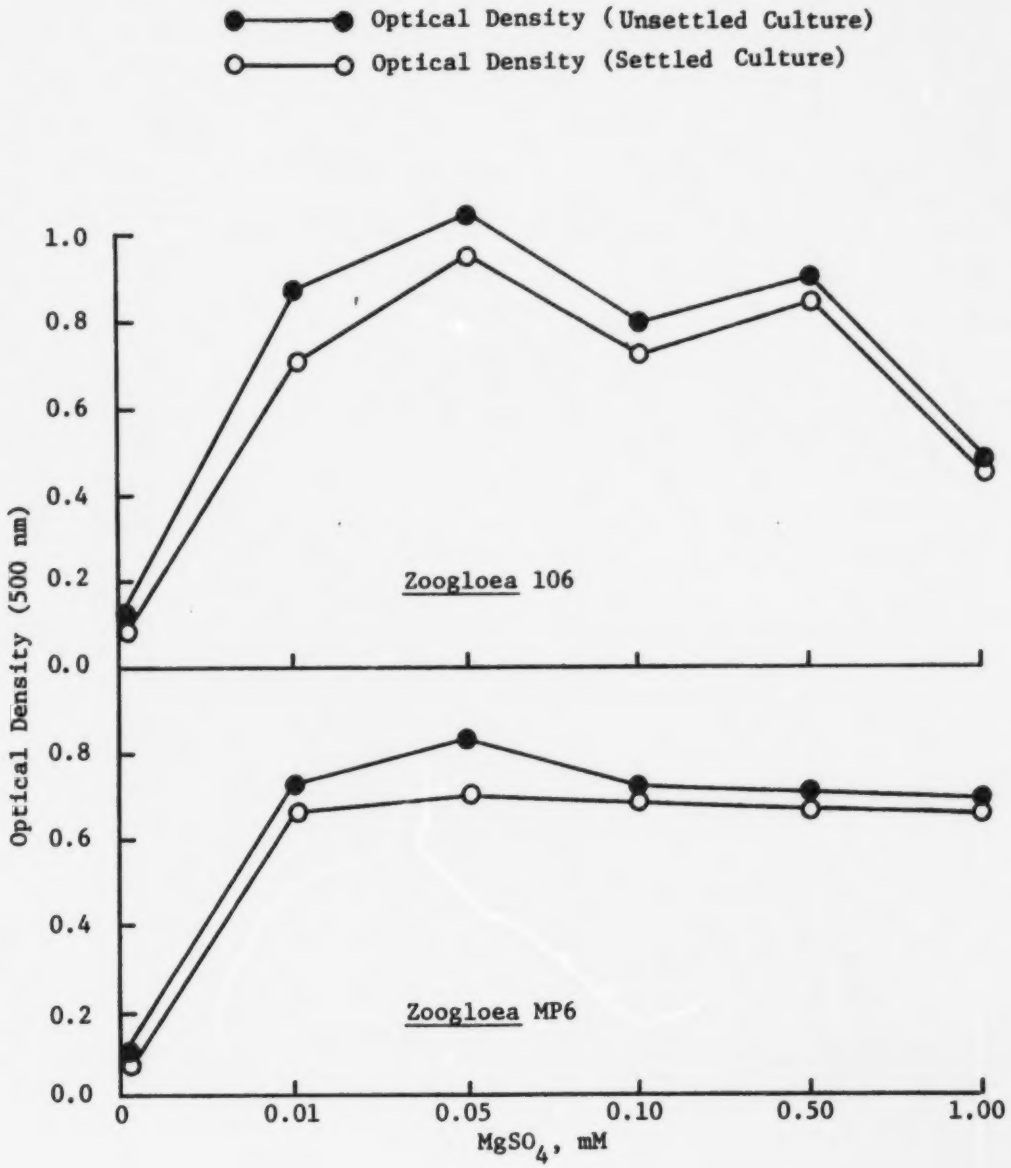

Fig. 26. Influence of Magnesium Ion on Flocculation of Zoogloea MP6 and Zoogloea ramigera 106.

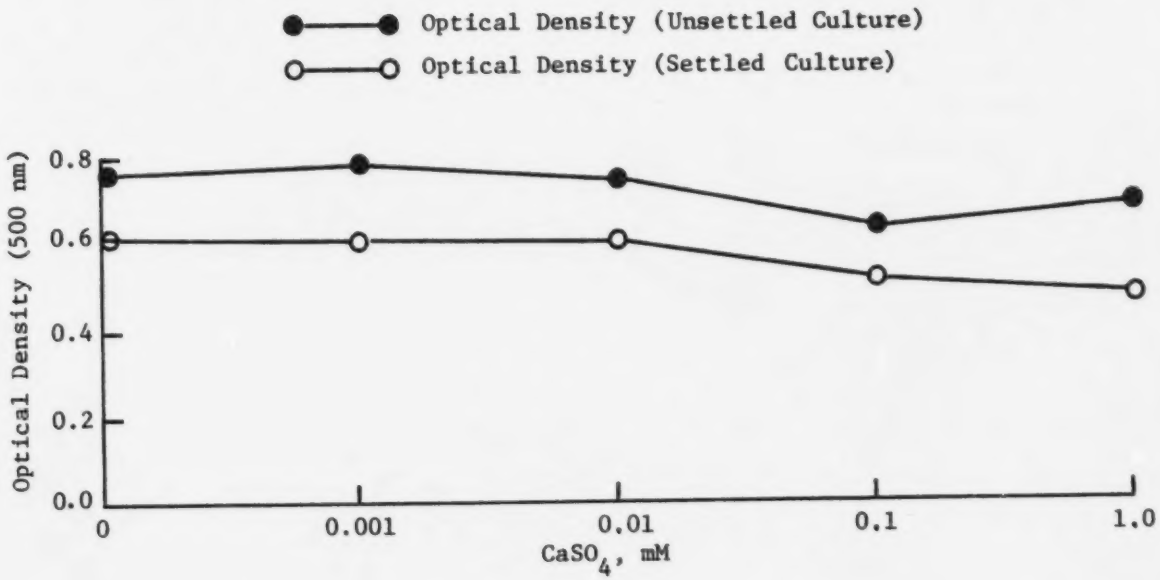

Fig. 27. Influence of Calcium Ion on Flocculation of Zoogloea ramigera 106.

Table 12. Influence of Metal Ions on Flocculation of Zoogloea MP6, 21 and Z. ramigera 106

Zoogloea strain no.	Cation[a]	Cation concentration (millimole/l)	Index of Flocculation[b] Experiment 1	Experiment 2
MP6	Ca^{++}	1.0	0.71	0.72
		0.1	0.47	NT[d]
		0.01	0.18	NT
		0.001	0.16	NT
		0.0	0.14	0.36
MP6	Mg^{++}	1.0	0.06	NT
		0.1	0.04	NT
		0.01	0.08	NT
		0.0	0.14	NT
MP6	Mn^{++}	1.0	--[c]	NT
		0.1	0.11	NT
		0.01	0.04	NT
		0.001	0.05	NT
		0.0	0.14	NT
MP6	Zn^{++}	1.0	--	NT
		0.1	--	NT
		0.01	--	NT
		0.001	0.01	NT
		0.0	0.14	NT
MP6	Na^{+}	1.0	0.05	NT
		0.1	0.06	NT
		0.01	0.10	NT
		0.001	0.07	NT
		0.0	0.14	NT
MP6	Fe^{+++}	1.0	(salt precipitated)	NT
		0.1	0.18	NT
		0.01	0.20	NT
		0.001	0.19	NT
		0.0	0.14	NT
106	Ca^{++}	1.0	0.25	0.72
		0.1	0.22	NT
		0.01	0.18	NT
		0.001	0.17	NT
		0.0	0.19	0.35

Table 12. (Cont'd)

Zoogloea strain no.	Cation[a]	Cation concentration (millimole/l)	Index of Flocculation[b] Experiment 1	Experiment 2
106	Mg^{++}	1.0	0.0	NT
		0.1	0.04	NT
		0.01	0.20	NT
		0.0	0.19	NT
21	Ca^{++}	1.0	0.21	NT
		0.0	0.09	NT

[a] All cations present as compounds of sulfate

[b] Index of Flocculation (I. F.) values determined from optical density measurements on settled and unsettled cultures after 48 hr incubation using the formula: $I.F. = \frac{O.D._{500}\ \text{Unsettled} - O.D._{500}\ \text{Settled}}{O.D._{500}\ \text{Unsettled}}$

[c] -- indicates cation was toxic

[d] NT indicates cation was not tested

However, similar agitation of nonflocculating cultures resulted in a dispersion of the cells in the surface film making the culture fluid strikingly turbid. Spread plating serial dilutions of each type of culture gave rise to two colonial forms. The "rough" colony was raised with a rugose surface whereas the "smooth" colony was slightly raised with a smooth surface (Fig. 28). Rough colony forming bacteria were present in major proportions in flocculating cultures of Zoogloea MP6 and were only minor in nonflocculating cultures (Table 13). When separate cultures developed from single rough and smooth colonies were agitated on the gyrotary shaking machine, only the rough type culture demonstrated flocculation (Table 14).

Temperature and flocculation

Zoogloea MP6 increased rapidly in cell mass when cultured on trypticase soy broth at 28 C and 36 C and more slowly at 20 C. Bacterial zoogloeae were formed at all temperatures. Exopolymer production, based on amino sugar analysis of purified zoogloea matrix, paralleled the increase in dry weight (Fig. 29) and it could not be determined that polymer production was especially favored at any of the temperatures tested.

Reducing agents and flocculation

In separate trials, various concentrations of sodium thioglycollate, sodium ascorbate, and sodium thiocyanate were incorporated in agar underlayers in beakers containing lactate-mineral salts medium inoculated with Zoogloea MP6. Beakers were kept well covered to avoid contamination during incubation. Both sodium thioglycollate (Fig. 30) and sodium ascorbate (Fig. 31) were effective in depressing the oxidation-reduction potential and a zoogloeal film developed at the surface of the medium. The amino sugar content per unit of film dry weight increased at higher concentrations of sodium thioglycollate. Optical density values of culture fluids were lower at the higher concentrations of sodium thioglycollate and sodium ascorbate indicating a low density of dispersed bacteria in the culture medium below the surface film. Sodium thiocyanate did not effect lowering of the oxidation-reduction potential as did the reducing agents and the dry weight of the surface film was less at any concentration of sodium thiocyanate used than in control cultures (sodium thiocyanate absent). However, amino sugar per unit weight of film did increase with increasing concentrations of sodium thiocyanate (Fig. 32). In all cases, increased concentrations of any of the three sodium salts in the agar underlayer resulted in cells becoming stratified closer to the surface of the culture medium indicating, possibly, that a toxicity gradient existed in the supernatant. As such, toxicity of the chemical agents rather than oxidation-reduction potential may have been the influential factor in determining the thickness of the surface film formed and the vertical range in the liquid medium within which cell proliferation occurred.

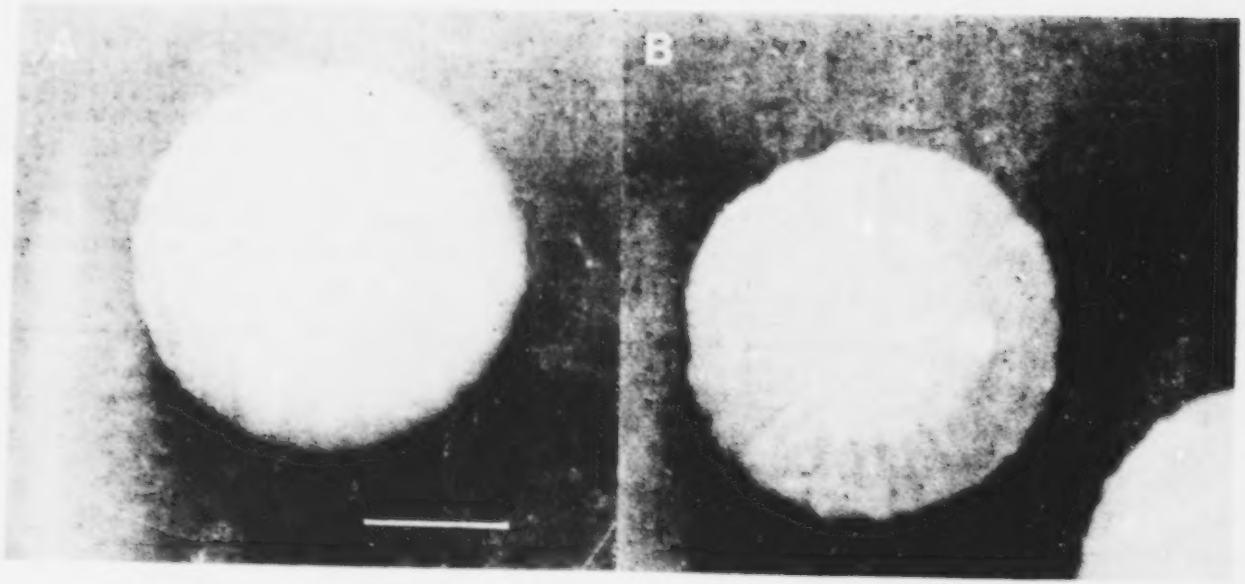

Fig. 28. Colonies of Zoogloea MP6 on Solid Culture Medium. A, smooth; B, rough. Sodium lactate-mineral salts culture medium; 5 days, 28 C. Photographed by reflected light. Bar equals 1.0 mm.

Table 13. Flocculation and Colonial Morphology (Solid Culture Medium) of Zoogloea MP6

Flask No.[a]	Optical Density of 48 hr culture		Index of Flocculation[b]	Number of colonies examined	Number of rough colonies observed	Percent rough colonies
	Initial	Settled				
1	0.49	0.25	0.49	232	216	93
2	0.46	0.43	0.07	155	38	24

[a] Growth medium was the basal medium (BM) with sodium lactate (1.0 g/l), culture flasks incubated at 20C on gyrotary shaker.

[b] Index of Flocculation (I. F.) values determined from optical density measurements on settled and unsettled cultures after 48 hr incubation using the formula:

$$\text{I. F.} = \frac{\text{O.D.}_{500}\ \text{Unsettled} - \text{O.D.}_{500}\ \text{Settled}}{\text{O.D.}_{500}\ \text{Unsettled}}$$

Table 14. Flocculation of Rough and Smooth Cultures of Zoogloea MP6[a]

Culture type	Optical density of 48 hr culture		Index of Flocculation[b]
	Unsettled	Settled	
Rough	0.45	0.22	0.51
Smooth	0.56	0.51	0.09

[a] Growth medium was the basal medium (BM) with sodium lactate (1.0 g/l); culture flasks incubated at 20 C on a gyrotary shaker

[b] Index of Flocculation (I.F.) values determined from optical density measurements on settled and unsettled cultures after 48 hr incubation using the formula:

$$\frac{O.D._{500}\ \text{Unsettled} - O.D._{500}\ \text{Settled}}{O.D._{500}\ \text{Unsettled}}$$

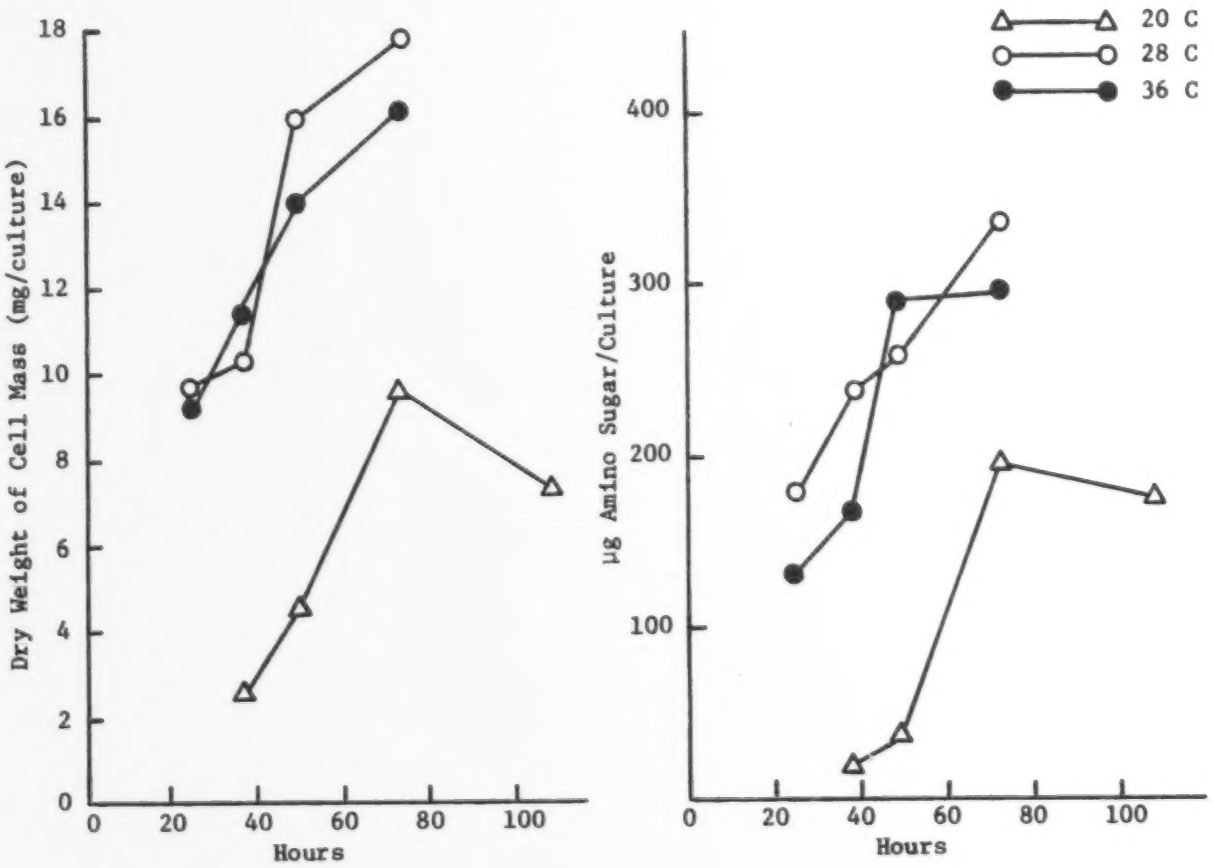

Fig. 29. Growth and Amino Sugar Production by Zoogloea MP6 at Different Incubation Temperatures. Static culture, trypticase-soy broth.

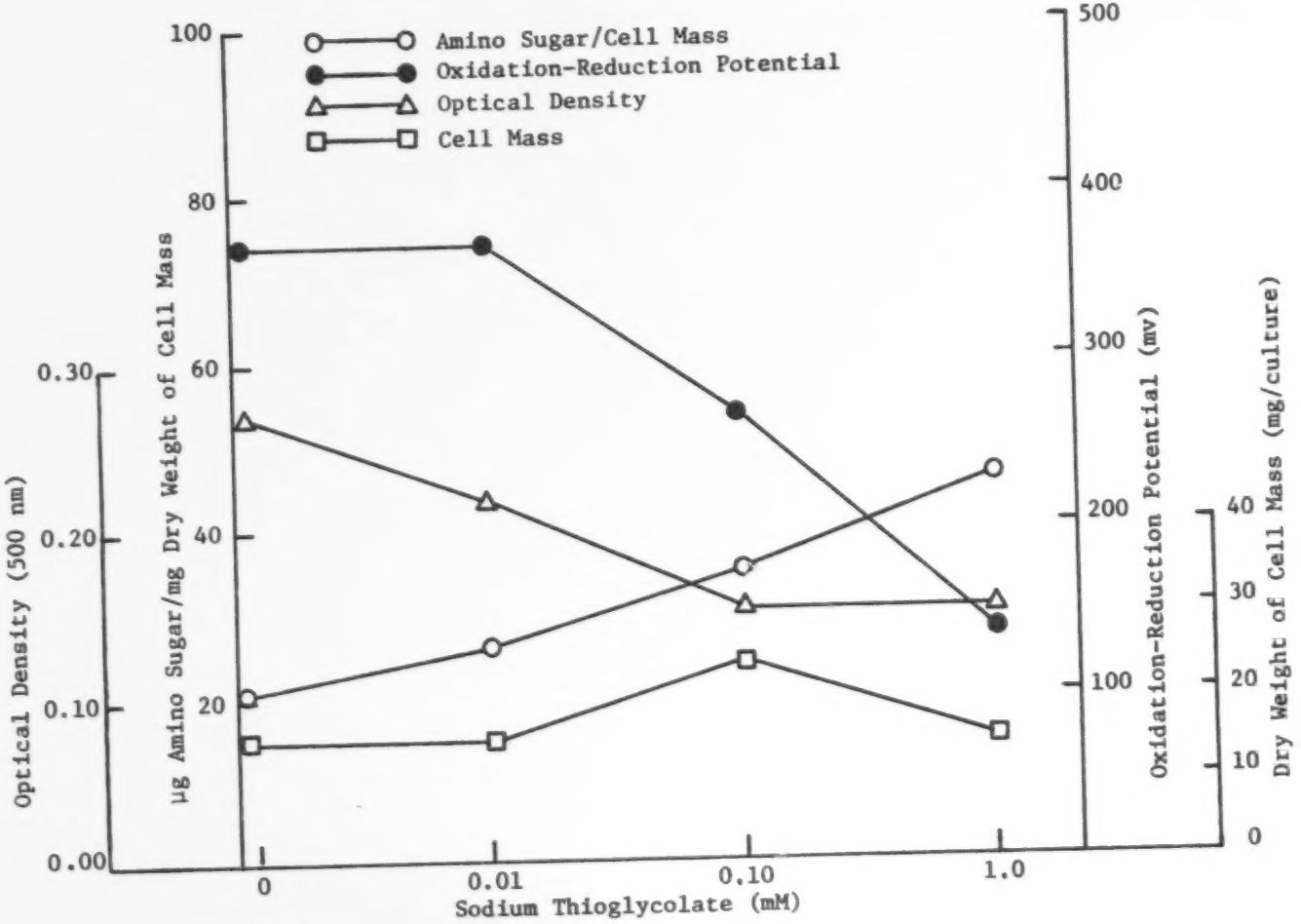

Fig. 30. Influence of Sodium Thioglycollate on Amino Sugar Production by Zoogloea MP6.

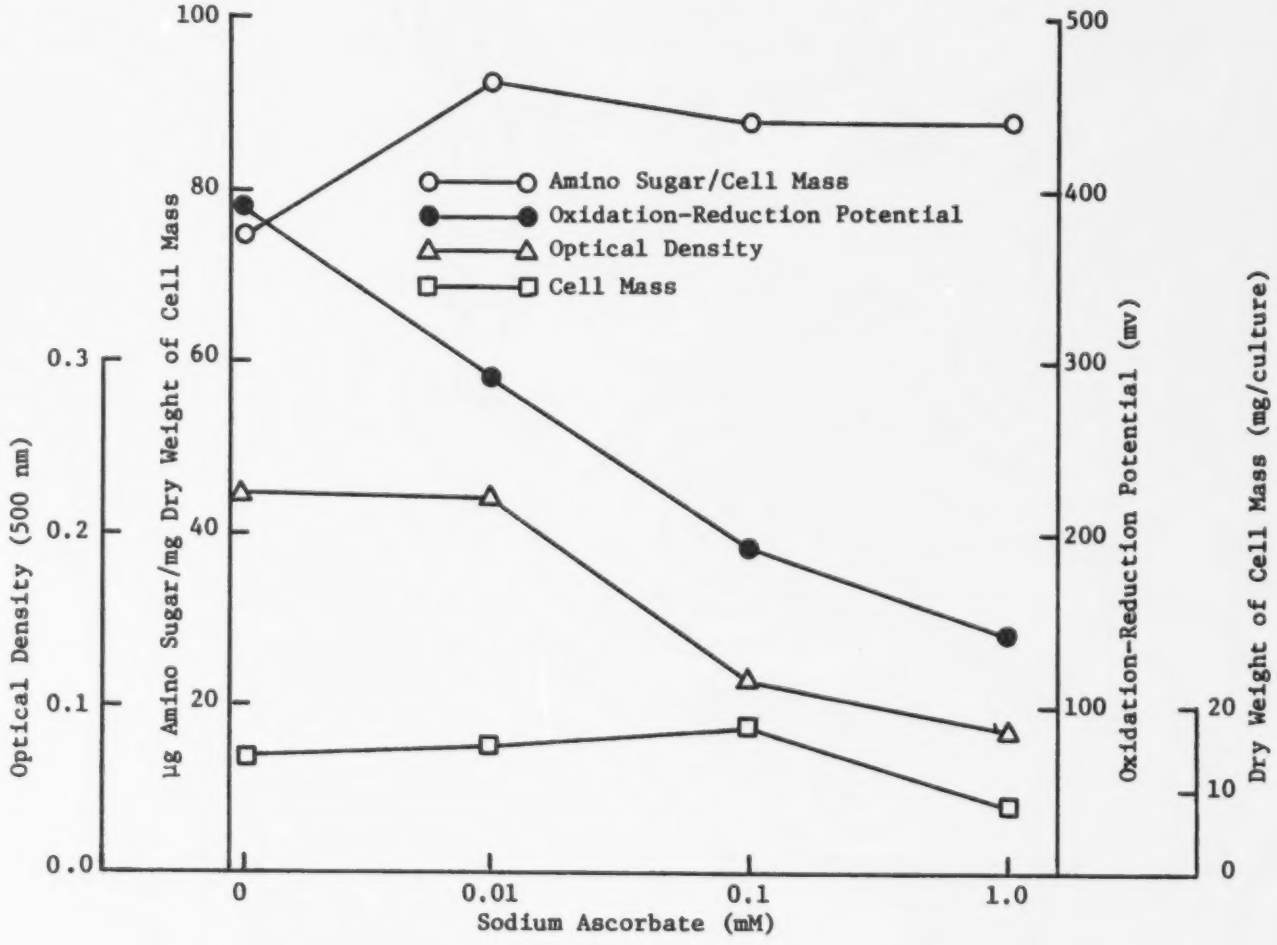

Fig. 31. Influence of Sodium Ascorbate on Amino Sugar Production by Zoogloea MP6.

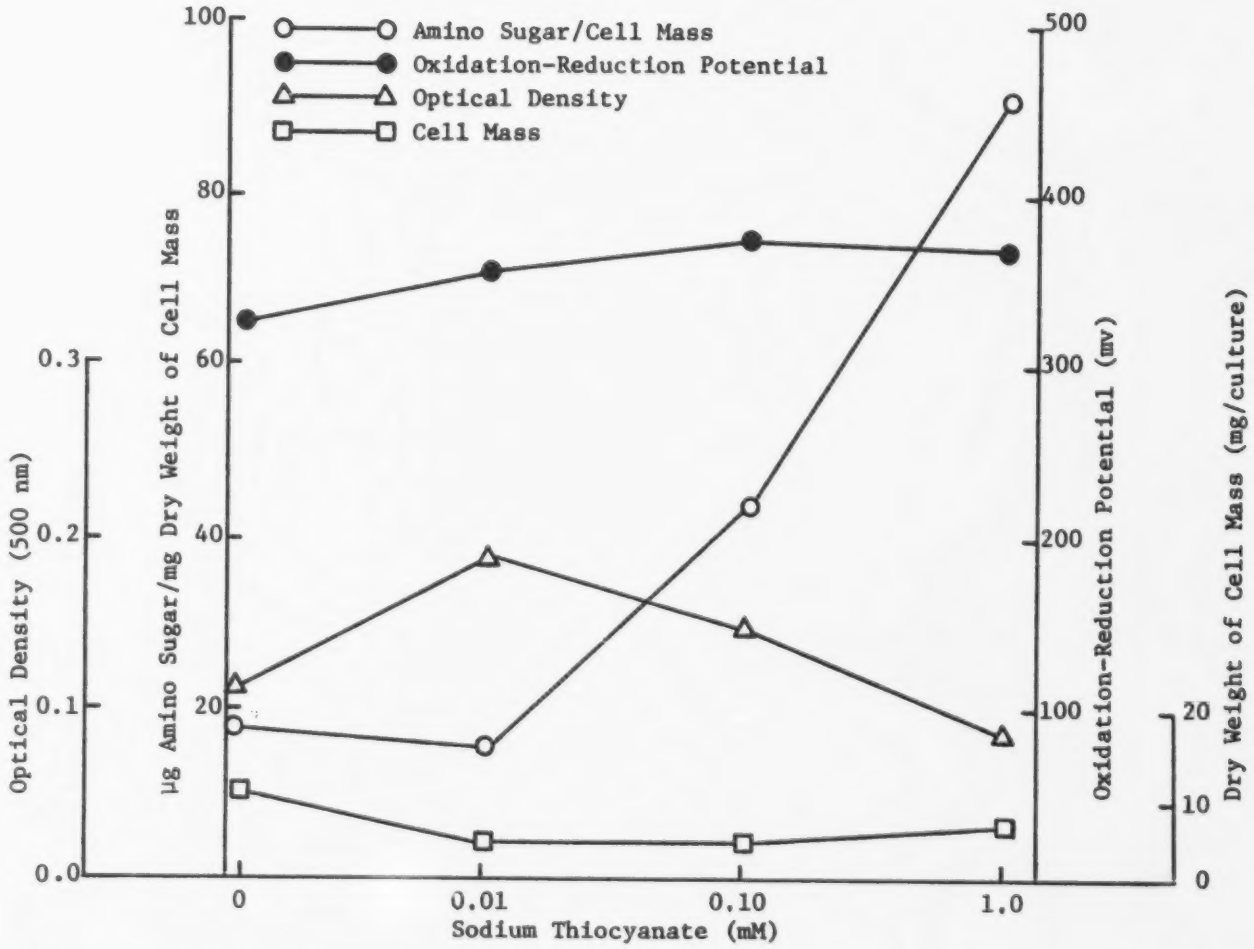

Fig. 32. Influence of Sodium Thiocyanate on Amino Sugar Production by Zoogloea MP6.

Carbon to nitrogen ratios and flocculation

Cells of Zoogloea MP6 flocculated well at nitrogen concentrations which did not limit the extent of growth in respect to the available carbon (substrate). At nitrogen concentrations which prevented maximum biomass production, index of flocculation values decreased considerably (Table 15). Flocculation could not be improved by increasing the nitrogen concentration of the culture medium above that required to attain maximum biomass. Decreased flocculation at low nitrogen concentrations may be related to reduced exopolymer production by the bacteria. At least the amino sugar to dry weight of culture ratios were much lower under these conditions than when higher concentrations of nitrogen were available in the culture medium. Varying the carbon content of the culture medium when providing a high initial nitrogen concentration did not affect the amino sugar production and index of flocculation values were consistently high.

NATURAL BACTERIAL ZOOGLOEA FORMATION

Mixed liquors stored in beakers for 48 hr at 28 C underwent changes in chemical and bacteriological characteristics, primarily: (a) lowering of the oxidation-reduction potential, (b) increase in volatile acid content, and (c) development of a scum with natural zoogloeae at the surface. The extent to which these effects developed seemed influenced by the mixed liquor suspended solids (MLSS) content of the mixed liquor, strength of the wastewater upon which the solids were produced or a combination of these. Mixed liquor obtained from the State College biooxidation basin, which receives a strong, unsettled domestic wastewater, exhibited a sharply lowered oxidation-reduction potential and an increased volatile acid concentration during storage. Increasing the MLSS content of the mixed liquor in beakers resulted in an increased rate of change both in oxidation-reduction potential and volatile acid content (Fig. 33A). The scum which formed at the surface of the mixed liquor contained exopolymer produced by the zoogloea-forming bacteria and it was found that both amino sugar and dry weight of scum also increased when the mixed liquor had been supplemented with additional MLSS prior to storage (Fig. 33B). Mixed liquor obtained from the University Park activated sludge aeration basin, which receives a moderate strength primary effluent, remained essentially unchanged during storage unless the solids content of the mixed liquor was significantly increased prior to incubation in beakers (Fig. 34). The mixed liquor storage experiments were repeated using fresh samples of mixed liquor collected from the State College and University wastewater treatment plants and the results confirmed earlier findings, all of which are summarized in Table 16. It should be noted that the mixed liquor of the State College secondary biooxidation basin, which treats the effluent from the primary biooxidation unit, displayed no change during storage and it was not possible to initiate scum formation by increasing the solids content. Occasionally, however, small fragments of MLSS would collect at the surface of mixed liquor during storage and be inadvertently included in dry weight of scum determinations.

Table 15. Influence of Carbon to Nitrogen Ratio on Flocculation and Amino Sugar Production by Zoogloea MP6

Carbon as sodium lactate (g/l)	Nitrogen as ammonium sulfate (g/l)	Ratio of carbon to nitrogen	Index of Flocculation[a]	Dry weight of culture (mg/50 ml of culture fluid)	Amino sugar (µg/50 ml of culture fluid)	Ratio of µg amino sugar/ml dry weight of culture
0.32	0.55	0.6	0.50	10.9	440	41
0.32	0.28	1.1	0.48	10.8	390	36
0.32	0.11	2.9	0.62	13.2	460	35
0.32	0.028	11.4	0.68	9.3	320	35
0.32	0.011	29.0	0.31	5.1	80	16
0.32	0.0028	114.0	0.03	1.7	20	12
1.28	0.056	24.0	0.42	12.1	580	48
0.64	0.056	12.0	0.50	5.6	280	50
0.16	0.056	3.0	0.45	1.4	80	57
0.08	0.056	1.5	0.33	0.6	30	53

[a] Index of Flocculation (I. F.) values determined from optical density measurements on settled and unsettled cultures after 48 hr incubation using the formula:

$$\text{I. F.} = \frac{\text{O.D.}_{500}\ \text{Unsettled} - \text{O.D.}_{500}\ \text{Settled}}{\text{O.D.}_{500}\ \text{Unsettled}}$$

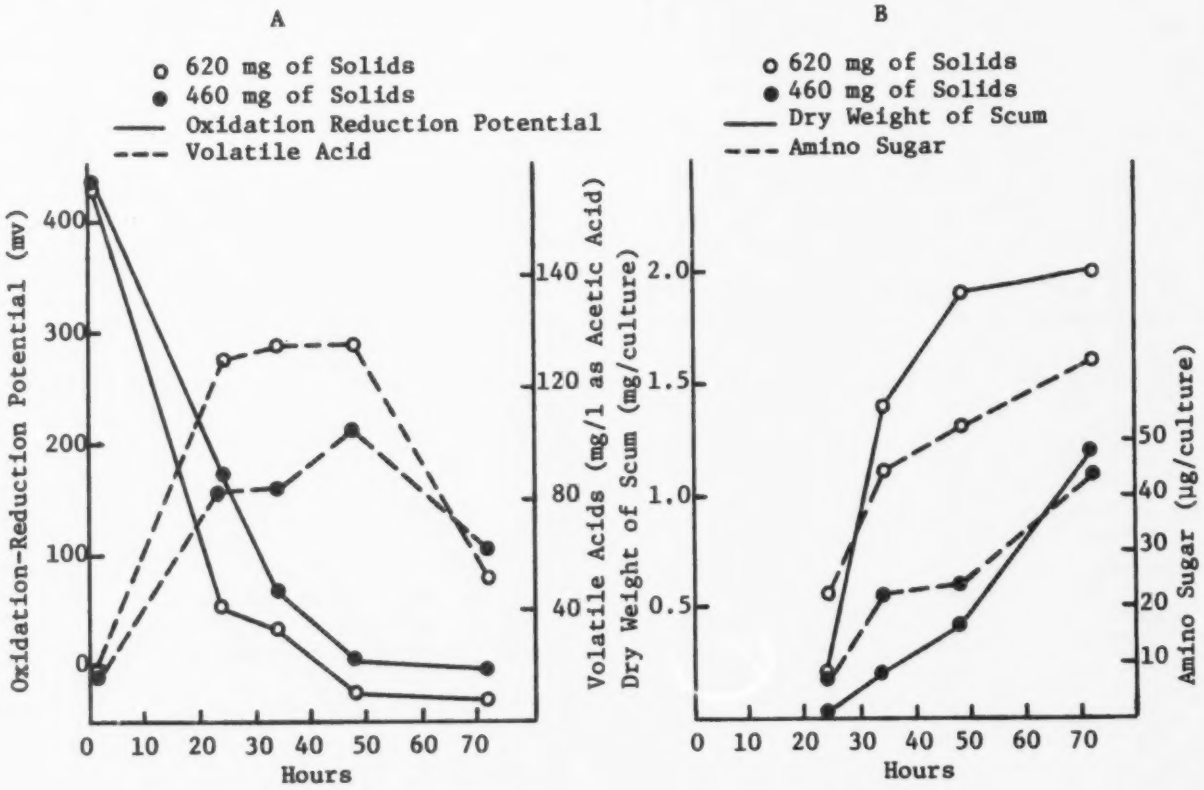

Fig. 33. Chemical and Microbiological Characteristics of State College Mixed Liquor During Storage. A, oxidation-reduction potential and volatile acids; B, dry weight of surface scum and amino sugar content.

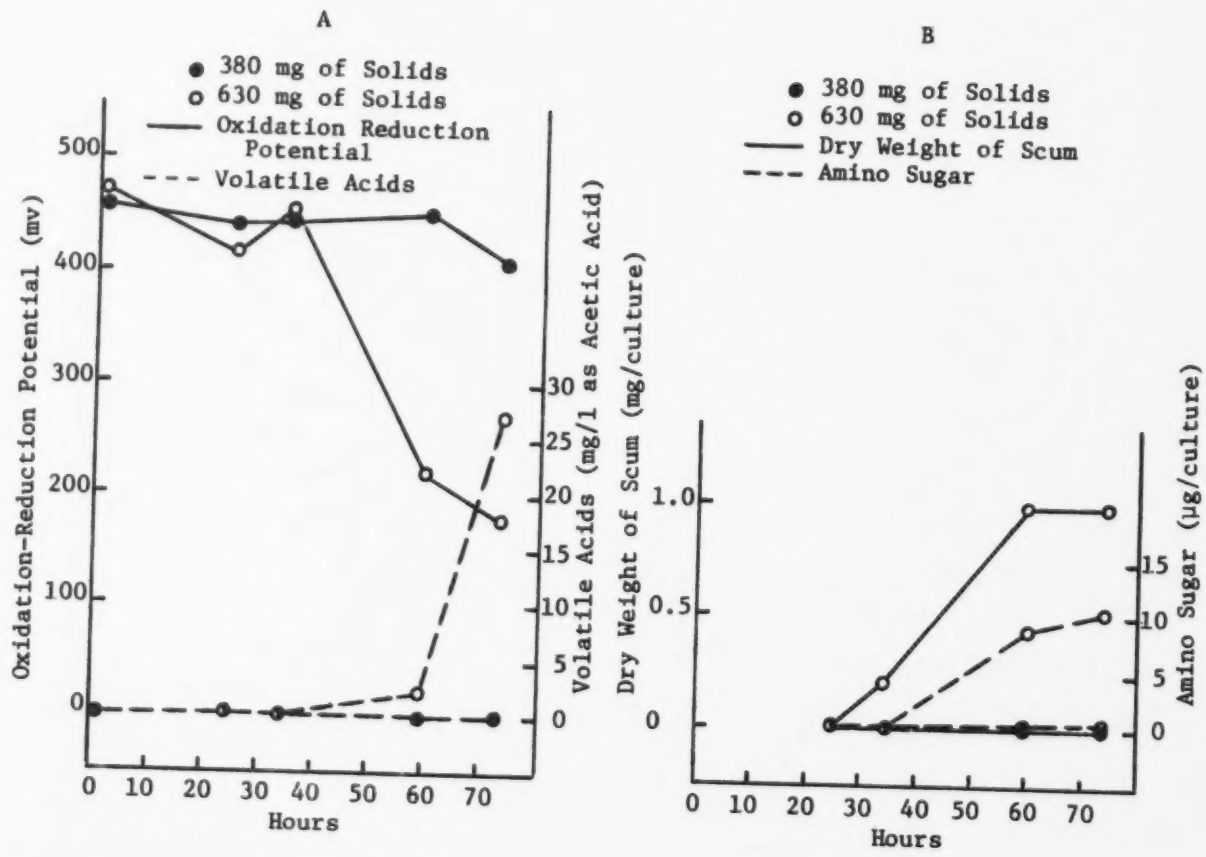

Fig. 34. Chemical and Microbiological Characteristics of University Park Mixed Liquor During Storage. A, oxidation-reduction potential and volatile acids; B, dry weight of surface scum and amino sugar content.

Table 16. Physical, Chemical, and Microbiological Characteristics of Stored Mixed Liquor[a]

Source of mixed liquor and sample letter		C.O.D. of influent wastewater (mg O_2/l)	Volume of mixed liquor stored (ml)	Volume of mixed liquor suspended solids stored (ml)	Weight of solids stored (mg dry weight	Oxidation-reduction potential of stored mixed liquor (mv) Initial	Final	Organic acids (mg/l as acetic acid)	Microbial scum mg dry weight	Microbial scum µg amino sugar
State College Aeration tank 1	-A	500	200	51	510	+490	-31	22	6.6	136
	-B	500	200	85	850	+490	-44	39	11.0	220
tank 2	-A	40	200	47	376	+490	+449	0	0.8	0
	-B	40	200	68	545	+490	+410	0	0.8	0
University Park Aeration tank	-A	180	200	43	382	+485	+407	0	0.0	0
	-B	180	200	71	632	+485	+177	27	1.0	11

[a] Mixed liquor stored in beakers for 72 hr at 28 C.

Since the appearance of a zoogloea-containing scum was regularly accompanied by increased volatile acids in the mixed liquor and lowered oxidation-reduction potential, additional studies were conducted to resolve the influence of each of these variables on scum formation. Agar containing a reducing agent was allowed to solidify at the bottom of a beaker before adding a volume of mixed liquor. The use of reducing agents alone to rapidly lower the oxidation-reduction potential did not promote scum formation in stored University Park mixed liquor. However, addition of sodium lactate to the mixed liquor prior to incubation regardless of whether or not a reducing agent was included, resulted in appreciable scum development which was measured in terms of dry weight and amino sugar content (Table 17). Oxidation-reduction potential was observed to decrease naturally with time in the absence of a supplementary reducing agent. In these experiments, scum formation took place only when sodium lactate was initially added to mixed liquor and was unrelated to the final value of the oxidation-reduction potential.

Eight substrates, in addition to sodium lactate, were tested for their ability to support scum formation in stored mixed liquor. Scums were harvested from each nutrient-enriched mixed liquor after 72 hr storage and placed in test tubes (Fig. 35). The dry weight and amino sugar content of the scum substance was determined (Table 18). Starch, glucose, sodium lactate, and sodium m-toluate visibly supported heaviest scum formation although, on the basis of amino sugar content, polymer production was enhanced by all nutrients except sodium o-toluate. Amino sugar generally increased with the dry weight of scum produced although the ratios of amino sugar to dry weight varied depending on the nutrient supplied. This variation may be expected since, in some cases, unavoidable inclusion of small portions of buoyant MLSS with harvested scum exaggerated scum dry weight values. All scums were heavily populated with finger-like zoogloeae. Although it was surprising to find that substrates such as starch and glucose supported relatively heavy development of finger-like zoogloeae, it may be that thse nutrients were decomposed by other micoorganisms in the mixed culture and that the zoogloeal bacteria utilized the metabolic depot products of nonzoogloeal organisms.

Table 17. Influence of Reducing Compounds and Sodium Lactate on the Formation of Scum at the Surface of Mixed Liquor Stored in Glass Beakers[a]

Reducing compound	Initial concentration of sodium lactate in the supernatant (mg/l)	Final oxidation-reduction potential (mv)[b]	Scum produced[b]	
			mg dry weight	µg amino sugar
None	0	+433	0.2	10
None	50	+48	6.4	126
None	200	-260	6.4	152
Sodium thioglycolate	50	-96	2.0	54
Sodium thioglycolate	0	+106	0.2	10
Sodium thiocyanate	0	+270	0.2	6
Sodium ascorbate	0	-344	0.2	6

[a] Each 600 ml beaker contained 200 ml University Park mixed liquor over 40 ml agar to which 1.0 millimole of a reducing compound was added.

[b] Analysis performed after 24 hr incubation of the mixed liquor at 28 C.; initial (time zero) oxidation-reduction potential for all cultures was essentially +432 mv.

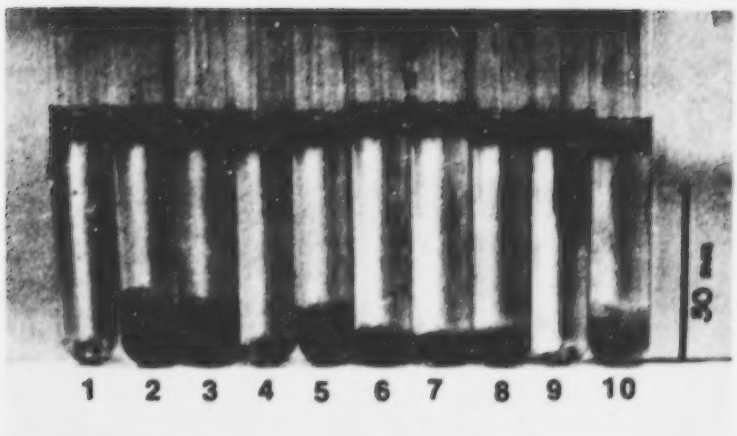

Fig. 35. Scum Layers Harvested from Beakers of Stored, Fortified Mixed Liquor. Nutrients added to mixed liquors: 1, none; 2, D-glucose; 3, starch; 4, D-xylose; 5, sodium lactate; 6, sodium acetate; 7, sodium glutamate; 8, sodium aspartate; 9, sodium o-toluate; 10, sodium m-toluate. Scums harvested after 72 hr incubation at 28 C.

Table 18. Influence of Organic Compounds on Scum Production at the Surface of Mixed Liquor Stored in Glass Beakers[a]

Substrate added	Concentration (mg/1)[b]	Final oxidation-reduction potential (mv)[c]	Scum produced[d]		µg amino sugar/ mg dry weight of scum
			mg dry weight	µg amino sugar	
None	--	+128	1.6	34	21
o-toluate	56	+200	1.2	34	28
starch	90	+97	4.3	148	34
glucose	90	+67	4.9	126	26
lactate	90	+38	5.0	126	25
m-toluate	56	+60	5.0	170	34
glutamate	108	+83	4.2	136	32
aspartate	156	+75	2.9	136	47
acetate	107	+80	3.9	90	23
xylose	90	+127	2.4	80	33

[a] Each 400 ml beaker contained University Park mixed liquor (430 mg suspended solids; 200 ml total volume of mixed liquor).

[b] Concentration of substrate added based on theoretical requirement of 3 millimoles O_2/1 for complete oxidation of the anion.

[c] Initial (time zero) oxidation-reduction potential for all cultures was essentially +441 mv.

[d] Harvested after 72 hr incubation at 28 C.

Section VI

DISCUSSION

The property of zoogloea formation is not restricted to any particular genus of bacteria. Examples of microorganisms capable of developing zoogloeae are Siderocapsa sp., certain autotrophic nitrifying bacteria, Azotobacter sp., Thiodendron sp., Thiocystis sp., and certain chemo-organotrophic pseudomonads, e.g., Zoogloea sp. Distinctive finger-like and dendritic zoogloeae originally described and assigned the binomial epithets, Zoogloea ramigera, by Itzigsohn (31) were first photographed by Koch (38). Koch envisioned that bacterial cells present in micro-colonies were so aligned that their proliferation gave rise to zoogloeae of peculiar shapes. Friedman and Dugan (23) contended that fingered zoogloeae resulted from an arrangement of individual packets of cells. It is evident from our studies that finger-like zoogloeae develop as a consequence of the unidirectional movement and multiplication of zoogloea-forming bacteria which may or may not originate in activated sludge flocs, microbial slimes and films, etc. It may be imagined that a marked coordination of cellular activities is required to produce these unusual zoogloeal structures, and, for this reason, it is anti-cipated that the formation of finger-like zoogloeae is restricted to the operations of a very few bacterial species. In effect, finger-like zoogloeae are the result of groups of bacteria able to move in a liquid environment while maintaining direction and colonial integrity. The movement of colonies of Bacillus circulans on solid medium is regarded as a rare activity among procaryotic organisms (39).

Results of fluorescent antibody experiments in which Z. ramigera 106 antiserum totally reacted with certain natural, finger-like zoogloeae lends support to our belief that the colonial structures are formed by bacteria of the genus Zoogloea. That many natural, finger-like zoogloeae did not react with the strain specific antiserum strengthens the view of others that several strains of Zoogloea probably exist in nature (23, 26). The absence of a cross reaction between Z. ramigera 106 antiserum and mixed cultures of filamentous, sheathed bacteria resembling Sphaerotilus sp. casts doubt on earlier suggestions that Z. ramigera is a growth form of Sphaerotilus sp.

Factors specifically responsible for stimulating the formation of finger-like zoogloeae remain unknown. However, it appears that the phenomenon involves an aerotactic or chemotactic behavior by the zoogloea-forming bacteria. The possibilities are that (a) oxygen limitations in flocs cause zoogloea-forming bacteria to grow outward in the direction of a higher oxygen tension, (b) concentrated waste products of floc bacteria induce zoogloea-forming bacteria to grow away from the floc and (c) active bacteria outside the floc produce substances which attract zoogloea-forming bacteria. At the least, it is visibly apparent that activated sludge flocs contain zoogloea-forming bacteria which

respond rapidly to environmental changes. The implications are that other, less obvious, changes in the activities and composition of the general sludge microflora may take place in response to altered environmental conditions. Although many studies have been conducted on the microbial composition of activated sludge (2,5,7,18,35,36,41,45,46,62, 69), little information is available to substantiate whether or not qualitative and quantitative changes regularly occur among the microorganisms in sludges. Some workers (1, 32) have compared the microbial composition of activated sludges at different stages of metabolism and age, however, their techniques would not be sensitive enough to reveal very subtle differences in sludge microflora over short intervals of time, e.g., semi-daily or less. Development of methods to analyze very carefully the stability as well as the composition of the microflora of activated sludge would greatly enrich the knowledge and remove some of the mystique attendant to biological wastewater treatment systems.

On the basis of our investigations of activated sludge flocs using microculture and fluorescent antibody techniques, it appears that the activated sludge process is not especially conducive to the formation of finger-like zoogloeae. As previously noted by Unz and Dondero (65), these zoogloeal structures are infrequently observed in fresh activated sludge flocs. Possibly, an occurrence of finger-like zoogloeae in fresh activated sludge reflects a change in wastewater composition or treatment plant operations. The appearance of zoogloeae at the surface of standing wastewater effluent is taken to be evidence of insufficient treatment in the so-called Kolkwitz "zoogloea test" (40).

The property of zoogloea formation may be advantageous to the pertinent bacteria in (a) protecting cells from predators, (b) maintaining large numbers of cells in a fixed location thereby assisting domination and survival of the organisms, (c) allowing adsorption and absorption of nutrients on the surface of the zoogloeal matrix which may also act to transfer soluble substrates and waste products from regions of higher to lower concentrations and, (d) incorporating other nonzoogloeal but biochemically active bacteria in the microbial assemblage. Unz and Dondero (67) isolated several genera of bacteria from natural, finger-like, wastewater zoogloeae which they found to be much more active in certain biochemical tests than Zoogloea sp. and suggested that the nonzoogloeal bacteria in the wastewater environment may act upon accumulated nutrients unavailable to Zoogloea sp.

Several publications have appeared in connection with the chemistry of the capsules and exopolymers of bacteria alleged to be zoogloeal (5, 13,24,52,60,70). Among these, amino sugars were referred to only three times. Anderson (5) isolated two monosaccharides from the exopolymer of a zoogloea-forming bacterium which he designated glucosamine and arabinose. Crabtree et al. (13) obtained hexosamines in hot water extracts of flocs and cells of Z. ramigera I-16-M. Most recently,

Tezuka (60) found two amino sugars in the exopolymer of a zoogloeal isolate and identified the compounds as N-acetyl glucosamine and possibly, N-acetyl fucosamine. We believe that the two amino sugars obtained by us in chemical analyses of the exopolymers of Zoogloea MP6 and Z. ramigera 106 are similar to those described by Tezuka except that we could not determine that the amino sugars were N-acetyl derivatives. However, unlike Tezuka, we specifically performed a chemical test for the N-acetyl groups. Nevertheless, our Zoogloea strains compare closely with the culture of Tezuka in several important cultural and physiological characteristics. Amino sugars appear to be a convenient class of substances to analyze for in determining indirectly the exopolymer content of cultures of Zoogloea sp. However, since amino sugars are only an indirect measure of exopolymer, it must be emphasized that this parameter will not be valid for all types of exopolymers including those of certain strains designated Zoogloea.

It is not surprising to find that the reducing substances and amino sugar content of activated sludge exopolymer is much lower percentagewise than in Zoogloea sp. exopolymer. As inferred by Busch and Stumm (9), sludge exopolymer is probably composed of the extracellular products of several types of resident bacteria, all of which are unique in certain characteristics. The presence of noncarbohydrate substances in the sludge polymer would diminish the amino sugar content of the polymer on a percentage basis. Others have reported on the existence of major components of a noncarbohydrate nature in sludges (37,49, 50, 53, 70) and the presence (70) and absence (22,62) of hexosamines and N-acethylhexosamines.

Our observation that intense flocculation by Zoogloea strains commences in late logarithmic growth phase confirms earlier results of Finstein (21). It appears that, depending upon culture conditions, zoogloea formation may result from a few encapsulated cells which coalesce and increase the size of the floc by merging with other cells, multiplication of individual polymer producing bacteria, and a combination of these. The former mechanism is more likely to occur in agitated cultures where random collision of cells is frequent. Under quiescent conditions, zoogloea-forming bacteria would be expected to develop zoogloeae primarily through multiplication and exopolymer production. We have repeatedly found that dispersed cells of Zoogloea strains are clearly encapsulated only during the flocculation phase. Others have commented on the lack of capsules on cells of zoogloeal bacteria (8,13,26).

Calcium ion has been found stimulatory (59,69), inhibitory (6) and neutral (58) in connection with the flocculation of microorganisms. In the present study, calcium either strongly enhanced the flocculation of cells of Zoogloea strains or had no effect. The nature of the floc promoting property of calcium ion cannot be described as mere electrostatic attraction since several other bivalent cations did not induce flocculation under any experimental conditions. Perhaps calcium ion

is involved in chelation with negatively charged groups on the exopolymer resulting in a bridge between bacterial cells. Peter and Wuhrmann (53) suggested interaction between microbially synthesized humic acids and polyvalent cations as an effective mechanism for the formation of activated sludge. The fact that calcium ion positively effects flocculation of cells of Zoogloea sp. which are capable of natural flocculation may be indirect evidence of the presence of certain negatively charged groups in the exopolymer necessary for chelation.

The discovery of smooth and rough colony-forming bacteria among strains of Zoogloea came too late to permit a detailed commentary on these organisms. It can be said that the presence of the two cell types in test cultures quite likely contributed heavily to the variation observed in flocculation experiments; a predominance of smooth type cells resulting in poor flocculation and, conversely, rough cells enhancing the flocculence of cultures. We have noted during several years of handling Zoogloea strains that freshly isolated cultures produced very little turbidity in the growth medium. However, with continuous subculturing, cultures became progressively more turbid and, in view of current observations on rough-smooth cell types, it may be that the loop transfer of culture encouraged the selection of smooth cells, since, generally, subculture inocula contained dispersed cells rather than flocs. In this regard, we recommend that freshly isolated cultures, presumed to be Zoogloea sp., be preserved by lyophillization and working cultures be frequently plated and examined for the appearance of rough and smooth type colonies whereupon each form may be maintained as desired. We have only begun to screen our collection of Zoogloea strains for rough and smooth colony forming cells. At this time, three cultures have been found to contain each cell type. Although rough forms of the bacteria very distinctly form cohesive flocs, it is not known if these are the predominant type in nature or to what degree they may revert to smooth type cells. Although smooth cells are, essentially, nonflocculating, they are capable of aggregating to some extent in quiescenct conditions and it may be that they simply do not produce sufficient exopolymer precursor and enough active enzyme to rapidly synthesize insoluble polymer to produce much floc. Such deficiencies in smooth cells may be the reason why calcium ion is not effective in their flocculation, that is, sufficient negatively charged groups are not present at the surface of the polymer to bind calcium ion and form tightly bridged cell aggregates.

It is difficult to speculate on the importance of the rough and smooth cells in the development of activated sludge since it is not known for certain if Zoogloea spp. have an active role in the formation of natural sludge flocs. On the other hand, assuming that zoogloea-forming bacteria are dominant among the polymer-producing sludge microflora and that smooth and rough types freely exist there, it is possible that the transition and behavior of the cells may influence the floc structure

and, consequently, the physical stability and settleability of the sludge solids. In addition, an abundance of polymer-producing cells in the sludge may contribute to extensive formation of sludge exopolymer, thus, increasing the nonviable matter in the sludge solids. Consequently, biochemical activity would diminish per unit of mixed liquor volatile solids. Although the sludge exopolymer consists of biodegradable sugar monomers, it cannot be construed that the exopolymer is readily decomposed by microorganisms since the difficult requirement to hydrolyze the glycosidic bonds contributes to the refractivity of the insoluble polymer. Nonzoogloeal, bacterial flocs may be disintegrated to individual cells by severe agitation whereas cells embedded in the gelatinous matrix of a zoogloea are held together and may be totally released only upon dissolving the exopolymer. Activated sludge contains both nonzoogloeal and zoogloeal bacteria and very likely, these are not uniformily distributed through out flocs.

Deflocculation of activated sludge is not well understood although nutrient deficiency may be a factor in its occurrence (56). Whatever the mode of action, microorganisms are responsible for the properties of activated sludge and it is the opinion of Busch and Stumm (9) that natural bioflocculation produces bacterial aggregates which are much less sensitive to shearing forces than flocs artificially established with the aid of synthetic polyelectrolytes.

It is not certain that reducing agents are able to enhance zoogloea formation by Zoogloea strains in axenic culture through lowering of the oxidation-reduction potential in culture fluids. Zoogloeal scums appear to form in static cultures of mixed liquors as a consequence of volatile acid production which is enhanced by reducing environments. Several of the short chain fatty acids which are normally formed from wastewater solids under anaerobic conditions have been found to be suitable nutrients for Zoogloea sp. (66). Under natural conditions, fatty acids may be generated in the form of depot products of anaerobic microorganisms acting upon organic matter. Diffusion of the volatile acids to regions of slight oxygen tension (microaerophilic zone) would permit them to serve as substrates for bacteria existing there. Unz and Dondero (65) have commented on the microaerophilic tendency of Zoogloea sp. which may also grow under definitely aerobic conditions.

We are encouraged that the exopolymer which we isolated from domestic activated sludge consisted of two amino sugars as did the exopolymer of Zoogloea strains. Our Zoogloea strains do not produce a microfibrilar or cellulase sensitive exopolymer as described in other studies (17,24,25). We are of the opinion that several kinds of bacteria exist in nature which are capable of producing zoogloeae and, in all probability, the chemical composition of the exopolymers vary considerably. However, in discussing Zoogloea sp., we take the position that the misidentification of bacteria presumed to be Zoogloea sp. has occurred

regularly in the past and will continue in this vein until the taxonomic status of the genus Zoogloea and its members is clearly defined. A reexamination of the genus Zoogloea and its species has been called for by Zvirbulis and Hatt (71). Contrary to suggestions by Friedman et al. (25), fibrilar polymers are not produced by all floc-forming bacteria and the entrappment of bacteria in fibrils cannot be regarded as the general basis upon which to explain natural bacterial flocculation. Furthermore, the property of exocellular microfibril synthesis is not universal among Zoogloea species and does not appear to be a useful taxonomic character of these organisms. The Zoogloea strains which we used for the major part of this study had the capacity to form finger-like zoogloeae in axenic culture and had been thoroughly characterized (65,66,68). These bacteria best represent the organisms responsible for forming typical, finger-like zoogloeae in wastewaters and the data obtained in this study should be treated accordingly.

Section VIII

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Section IX

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1. Unz, R. F. and Farrah, S. R., "Use of Aromatic Compounds for Growth and Isolation of Zoogloea," Applied Microbiology, 23, pp 524-530 (1972).

Section X

GLOSSARY

Activated sludge- A consortium of microorganisms, principally bacteria, assembled in flocculent form and developed through aeration of wastewaters.

Antibody- A specific proteinaceous substance synthesized in the body of an animal in response to the presence of an antigen.

Antigen- A foreign matter which when introduced into the body of an animal elicits the production of antibody.

Antiserum- Blood serum which contains antibodies.

Cinematography- Motion picture photography.

Exopolymer- An extracellular, macromolecular substance of microbial origin which constitutes the chemical basis of the gelatinous matrix of a zoogloea.

Finger-like zoogloea- A peculiar, elongated zoogloea in which the resident bacteria are generally arranged parallel to the longitudinal axis of the zoogloea.

Floc- A flaky or granular aggregation of microorganisms which may be cohesive or diffuse and may or may not be zoogloeal

Fluorescent antibody- Antibody which has been conjugated with a fluorescent dye to assist in the location of antigen-antibody complexes with the use of ultraviolet illumination and the microscope.

Mixed liquor- The mixture of activated sludge and wastewater which is aerated in the activated sludge wastewater treatment process.

Mixed liquor suspended solids- The suspended solids, mostly activated sludge, present in mixed liquor

Oxidation-reduction (redox) potential- A measure of the intensity level of a system to donate (reduction) or accept (oxidation) electrons.

Serology- A field of study specializing in serum and serum reactions.

Zoogloea- A structure which consists of microorganisms embedded in a confining gelatinous matrix. Not a generic epithet.

Zoogloea ramigera- Epithets for the genus and species of a legitimate bacterium.